UNIVERSITY OF LONDON THESIS

Degree PhD Year 2007 Name of Author FIDLER, Katherine Jane

The role of mannose binding lectin (MBL) in infection and inflammation

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Thesis submitted in fulfilment of the requirements

for the degree of PhD

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This work was supported by the Wellcome Trust (UK) through a Clinical Training Fellowship

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Declaration of Content

I declare that all of the work in this thesis has been undertaken by myself except where indicated below.

Signed.....

A) Clinical Studies and collaborations

Cystic fibrosis (CF) study

The first study involved a cohort of children with cystic fibrosis from Great Ormond Street Hospital, London, UK, in which I was wholly responsible for the ethics application, patient recruitment, sample processing and data analysis. The second was performed in collaboration with Dr Jane Davies (Senior Lecturer and Paediatric respiratory consultant) and involved a cohort of adults and children with CF, managed at the Royal Brompton Hospital (RBH), London, UK and recruited by Dr Jane Davies. In the second study I performed all of the work on the patients with *Burkholderia cepacia complex* and part of the DNA extraction, MBL haplotyping and discussion of results. Parts of these results, relevant to my own, are presented in this thesis. I was not involved in the clinical care of any of these patients and therefore "blinded" to their outcome until data analysis.

Bronchoalveolar lavage (BAL) study

I was responsible for the study design (in collaboration with Dr Jane Davies), ethics application, parental information leaflets & sample processing for the GOSH patients in this study. In addition I performed the bronchoalveolar lavage & serum MBL ELISA's on the additional BALs collected at the Royal Brompton Hospital. The neutrophil

elastase assay was performed by Dr Tom Hilliard, Clinical Research Fellow at the Royal Brompton Hospital. Data analysis for all the patients was done by myself.

PICU study

I was responsible for the study design, ethics application, parental information leaflets, sample processing and data analysis in this study. In addition Dr Mark Peters (Paediatric ICU consultant) and Dr Peter Wilson (Clinical Fellow, PICU) helped with patient recruitment and consent. Dr Peter Wilson compiled the database of clinical parameters. I performed the EndoCab ELISAs in conjunction with Dr Rob Stephens (Clinical Research Fellow, ICH) and the cytokine polymorphisms in conjunction with Dr Paul Kotwinski (Clinical Research Fellow, Rayne Institute, UCL). The technique for the detection of the IL-10 -1082 (G/A), TNF-α -308 (G/A), ACE and PAI 1 promoter polymorphisms had been optimised prior to the onset of this study by Dr Meredith Allen (Clinical Research Fellow at ICH) and Dr Paul Kotwinski.

General

I acknowledge the help of the nursing and medical clinical staff and the patients and their parents/carers in all of these studies.

B) Laboratory work

All other laboratory work presented here is entirely my own. Kind donations of any materials or compounds are acknowledged in the relevant section.

ABSTRACT

From early in life humans come into contact with a wide variety of infectious agents including bacteria, fungi and viruses. However, despite the vast array of potential pathogens, only a minority have the capacity to cause disease in a human host. This is largely due to the efficacy of the human innate and adaptive immune systems. The innate immune system consists of a variety of components including mechanical barriers, secretions, cells, excreted proteins, and serum proteins including the complement components and mannose binding lectin (MBL). MBL is a liver derived, acute phase, circulating serum protein that acts as a pattern recognition molecule. It is able to bind to a range of sugars presented in particular conformations on the surface of microbes and, having bound to its target, it can activate the lectin complement pathway and enhance complement-independent opsonophagocytosis. Over the last decade the biological importance of MBL has become increasingly apparent as indicated by the clinical consequences of the MBL deficient state. This has mainly been addressed by looking at the effect that MBL deficiency has on the susceptibility to a wide range of infections. More recently, interest has focussed on the role that MBL may play in the modulation of inflammation.

It is well recognised that individuals differ in their susceptibility to, and severity of, both infectious and non infectious diseases. This raises the question, could the role of inherited factors governing host response to infection and inflammation be important? The work described in this thesis investigates the role of MBL in two diseases that both involve an infective and inflammatory component, namely children with the chronic disease cystic fibrosis (CF) and those with acute inflammation/sepsis in intensive care.

In clinical studies I show that MBL deficiency is associated with worse pulmonary function tests in adults with CF and an increased risk of death and/or lung transplantation in children with CF. The reasons for this were explored by examining MBL binding to bacterial pathogens seen in CF and by examining bronchoalveolar lavage (BAL) fluid from children with and without CF. Here I show that MBL can be detected in the BALs of children with both acute and chronic respiratory disease but not in the controls. In another study of 142 children admitted to paediatric intensive care, I demonstrate an association between MBL deficiency and the development of the systemic inflammatory response syndrome and an increased severity of sepsis. This effect of MBL is independent of age, sex, ethnicity, polymorphisms in the genes for TNF-α, IL-6, IL-10, angiotensin converting enzyme (ACE), plasma activator inhibitor 1 (PAI-1) and levels of antibodies to endotoxin.

The work presented here highlights the importance of MBL in susceptibility to infection and may demonstrate a role for MBL in modifying the inflammatory response. These data may assist in furthering the understanding of mechanisms in disease pathogenesis as well as paving the way for exploratory research into MBL as a potential therapeutic agent.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor Nigel Klein and Dr Jane Davies for their guidance, support and encouragement throughout the extended course of this project. I am also greatly indebted to Professor Mac Turner and Dr Mark Peters for their critical appraisal of my work. In addition I would like to thank all those who collaborated with me, especially Dr Peter Wilson, Dr Tom Hilliard and Dr Paul Kotwinski, the numerous members of the immunobiology and infectious diseases departments for their patience and humour and the staff and patients without whom clinical research is not possible.

I would also like to thank my parents, especially my mother, and grandparents who bought me up with love and a desire to ask questions, and Penny, Michael and friends for always listening.

Most importantly I want to thank Martin for all his love and laughter and Arran, Euan and Polly who have all happily appeared during the course of this work and made our lives so much more fun!

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ABBREVIATIONS

ACE	Angiotensin converting enzyme
ALSPAC	Avon Longitudional Study of Parents and Children
BAL	Bronchoalveolar lavage
Bcc	*Burkholderia cepacia* complex
$\chi 2$ test	Chi square
CF	Cystic Fibrosis
ΔF508	delta F 508, commonest mutation in cystic fibrosis
GOSH	Great Ormond Street Hospital
IL-6	Interleukin - 6
IL-10	Interleukin - 10
KW	Kruskal-wallis
MADGE	Microtitre array diagnonal gel Electrophoresis
MBL	Mannose binding lectie
Pa	*Pseudomonas aeruginosa*
PAI-1	Plasminogen Activator Inhibitor - 1
PCD	Primary ciliary dyskinesia
PBS	Phosphate buffered saline
PI	Protease Inhibitor
PICU	Paediatric Intensive Care Unit
PIM	Paediatric Index of Mortality
PP	Pneumonia/pneumonitis
RBH	The Royal Brompton Hospital
RLRTI	Recurrent lower respiratory tract infection
RSV	Respiratory syncytical virus
SIRS	Systemic Inflammatory Response Syndrome
SOFA	Sequential Organ Failure Assessment
TNF-α	Tumour Necrosis Factor - α
TBS	Tris buffered saline
UA	Upper airway

CHAPTER 1

Introduction

1.1 Mannose-Binding Lectin (MBL) general considerations

In 1968, a child with failure to thrive, diarrhoea and frequent infections, with a serum dependent defect in phagocytosis of Baker's yeast (*Saccharomyces cerevisiae*) was described (Miller et al., 1968). In 1972 this defect was shown to be present in a series of children with unexplained frequent infections (mainly bacterial) and could be corrected, in vitro, by normal plasma suggesting that the cause was a defective factor rather than an inhibitor (Soothill and Harvey, 1976). Independently, a protein that could bind mannan particles from *Saccharomyces cerevisiae* was discovered in rabbit liver and termed mannose binding lectin (MBL) (Kawasaki et al., 1978). This was subsequently shown to be present in human serum (Kawasaki et al., 1983) and in 1989 Professor Turner's group established that deficiency of MBL was responsible for the opsonic immune defect first described in children (Super et al., 1989). The same year the human MBL gene (*mbl-2*) was sequenced independently by two groups (Sastry et al., 1989;Taylor et al., 1989) and identification of the first structural mutation, causing low protein levels, followed two years later (Sumiya et al., 1991).

Mannose binding lectin (MBL) is a liver derived, acute phase, circulating serum protein that acts as a pattern recognition molecule and plays a pivotal role in innate immunity. This role seems particularly important in the first few minutes after microbial invasion and in young children from 6-18 months, a vulnerable age during which passively transferred maternal antibody has largely disappeared but the adaptive immune response

is still immature, hence the coining of the term "*ante*-antibody" by Ezekowitz (Ezekowitz, 1991). MBL has now been found in all mammals and birds studied to date (Holmskov et al., 1994) and is estimated to have been present for the last five hundred and fifty million years since key elements of the innate immune system were developing in early invertebrates (Flajnik, 1998). Two distinct MBL proteins (MBL A and C) are found in many mammals such as rats and mice but only one is present in human serum. For the rest of this thesis MBL will refer to the human form unless specifically stated.

MBL is able to bind to a range of sugars presented in particular conformations on the surface of microbes but not to human proteins or normal cells (Weis and Drickamer, 1994;Sheriff et al., 1994) and therefore seems to belong to an ancient group of molecules important in the recognition of 'pathogen-associated molecular patterns' (PAMP's). Having bound to its target it can activate the lectin complement pathway (Ohta et al., 1990) and enhance complement-independent opsonophagocytosis (Kuhlman et al., 1989;Hartshorn et al., 1993;Tenner et al., 1995;Tenner et al., 1995). In addition it seems that MBL may play a role in the modulation of inflammation (Jack et al., 2001b) and the promotion of apoptosis (Stuart et al., 2006). Over the last seventeen years the biological importance of MBL has become increasingly apparent as indicated by the clinical consequences of the MBL deficient state (Super et al., 1989;Garred et al., 1995;Summerfield et al., 1995;Summerfield et al., 1997;Garred et al., 1999b;Koch et al., 2001;Fidler et al., 2004;Gordon et al., 2006) (see section 1.6).

1.2 MBL Structure

Lectins are proteins which bind to specific carbohydrate structures, but which are not enzymes or immunoglobulins (Barondes, 1988). MBL is a member of a group of lectins called the collectins, characterised by both a lectin and a collagenous domain (Malhotra et al., 1994b). Other members of the collectin family include the lung surfactant

proteins SP-A and SP-D in humans and bovine conglutinin and CL-43, all of which share similarities in structure. In particular MBL, SP-A and SP-D are large macromolecules and on electron microscopy both MBL and SP-A have a bouquet-like structure similar to C1q (Lu, 1997) (Fig 1.1). The basic subunit or monomer of MBL is formed from three identical polypeptide chains, each of approximately 32 kDa, that associate to form higher molecular weight oligomeric structures that range from dimers to hexamers (Turner, 2003), with trimers and tetramers being the most common in human serum (Lipscombe et al., 1995;Teillet et al., 2005). Each polypeptide chain contains four domains: (i) an N-terminal region comprising 21 amino-acids that is cysteine rich and is involved in protein oligomerisation by the formation of intra and inter subunit disulphide bonds, (ii) a collagenous domain that forms a classical triple helix and contains 59 amino-acids consisting of 20 tandem repeats of Glycine-Xaa-Yaa (except repeat 8 which consists of only Glycine-Glutamine), (iii) an α-helical hydrophobic coiled neck region that consists of 30 amino-acids and is crucial for the initiation of oligomerisation and (iv) a 188 amino acid carbohydrate recognition domain (CRD). This carboxy terminal CRD, in the presence of calcium, is able to bond to the 3- and 4-hydroxyl groups of certain sugars found on the surface of microbes, thus MBL acts, effectively, as a universal antibody (Turner, 1996;Garred et al., 2006) (Fig 1.2). The three sugar binding sites of the CRD of human MBL are separated by a distance of 45Å (Sheriff et al., 1994;Chang et al., 1994) and are all situated on the same face of the molecule giving a relatively flat binding platform for the binding of the repeating patterns of sugar groups found on microbial surfaces. Human mannose-type sugars are not however displayed at the same density therefore minimizing the chance of MBL binding to self proteins (Sheriff et al., 1994). Although the binding of each lectin to sugar is of low affinity (approximately 10^{-3} M) the binding of multiple sites can

achieve significant functional avidity. The recognition functions of the protein are mediated by the lectin domain and effector functions by the collagenous region, however it seems that for full functional activity of both parts that high order oligomeric structures such as tetramers are required (Yokota et al., 1995).

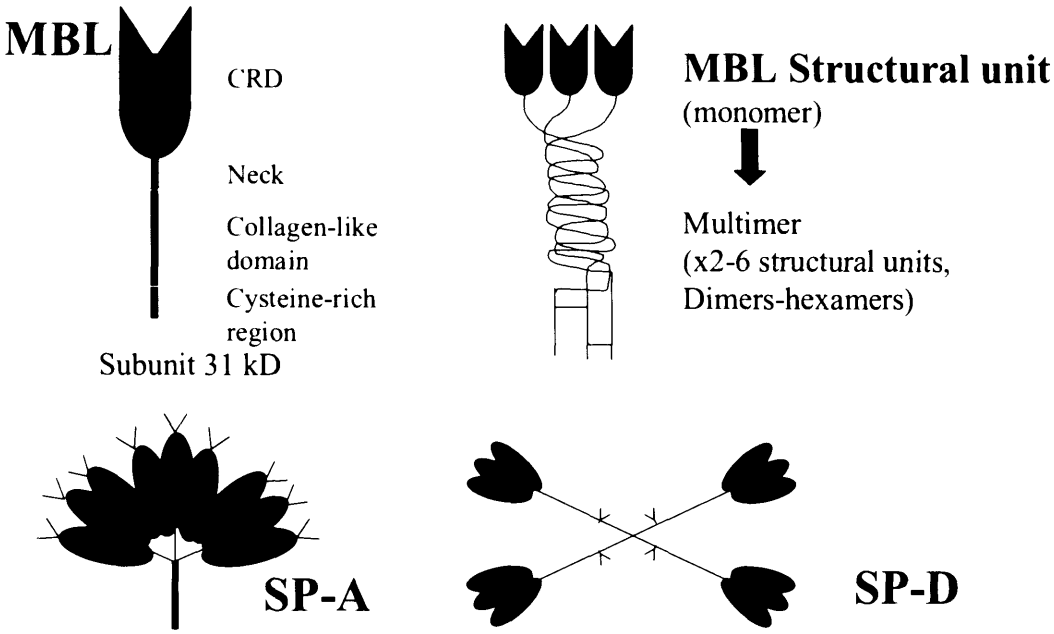


Fig 1.1 The collectin family of proteins

MBL, SP-A and SP-D are large macromolecules and on electron microscopy both MBL (see next Fig 1.2) and SP-A have a bouquet-like structure similar to C1q (Lu, 1997).

(Fig adapted from Prof. Klein with permission).

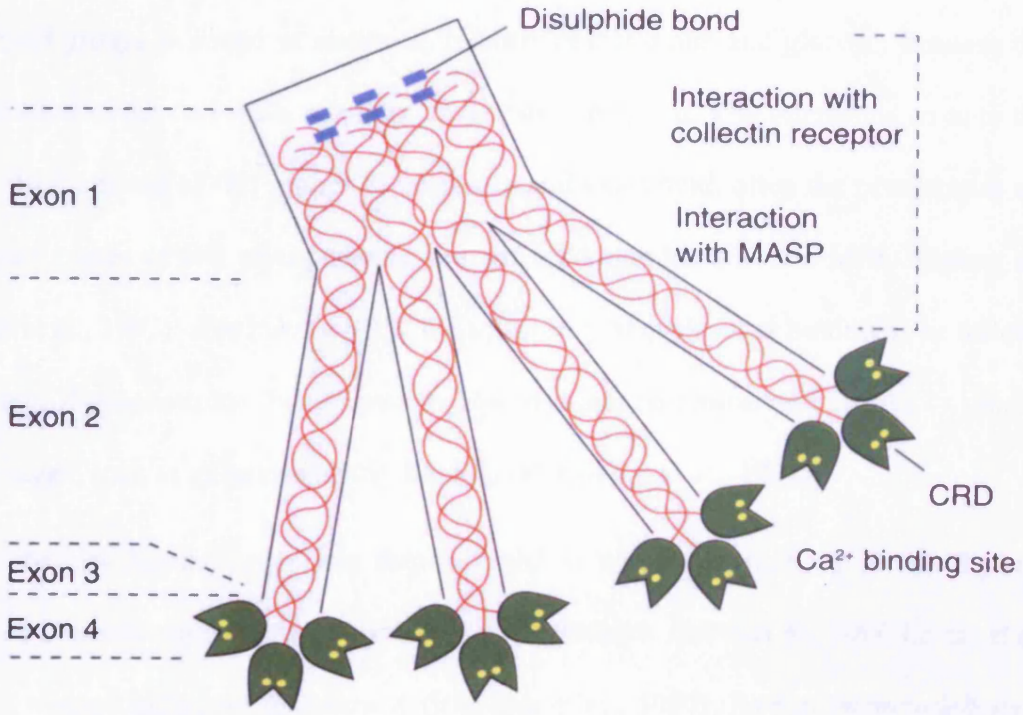


Fig 1.2 Mannose-Binding Lectin Structure

Representation of tetrameric MBL. The N-terminal region is seen at the top of the diagram with the C-terminus at the bottom. The CRD's contain Ca^{2+} dependent binding sites (represented as yellow spots). The four exons of the human MBL-2 gene encode for the protein regions indicated. (CRD: carbohydrate recognition domain). Taken from Turner, 1996, with permission.

1.3 MBL Function

1.3.1 MBL binding to micro-organisms

MBL binds to a wide range of micro-organisms that display certain sugars on their surface. It has been shown that a specific equatorial orientation of the C3 and C4-OH groups of these oligosaccharide ligands is required for MBL binding. This orientation of hydroxyl groups is found in mannose, N-acetylglucosamine and glucose; hexoses that are present in the cell walls of many infectious agents, but it is interesting to note that the configurations of OH groups in galactose and sialic acid, often the penultimate and ultimate sugars of self glycoproteins, are not accommodated by the MBL binding site (Weis et al., 1992). For human MBL the selectivity of such sugar binding is as follows: N-acetyl-D-glucosamine > mannose > N-acetyl-mannosamine and fucose > glucose with sugars such as galactose hardly binding (Holmskov et al., 1994).

Over the last decade, organisms demonstrated to bind to MBL have included yeasts such as *Candida albicans* and *Cryptococcus neoformans* (Neth et al., 2000;Levitz et al., 1993), viruses including *influenza A* (Reading et al., 1997), *human immunodeficiency* virus (HIV) (Ezekowitz et al., 1989;Haurum et al., 1993) and *herpes simplex* virus (Fischer et al., 1994), proteins from the malaria-causing parasite *Plasmodium falciparum* (Klabunde et al., 2002) and a large number of bacteria (Neth et al., 2000;Townsend et al., 2001;van Emmerik et al., 1994;Davies et al., 2000a). In one of the largest of these studies, binding of purified MBL to pathogens was investigated by flow cytometry. Diverse *Candida species*, *Aspergillus fumigatus*, *Staphylococcus aureus*, and *beta-haemolytic group A streptococci* exhibited strong binding of MBL, whereas *Escherichia coli, Klebsiella species*, and *Haemophilus influenzae* type b were characterized by heterogeneous binding patterns. In contrast, *beta-haemolytic group B streptococci, Streptococcus pneumoniae,* and *Staphylococcus epidermidis* showed low

levels of binding. Bound MBL was able to promote C4 deposition in a concentration-dependent manner (Neth et al., 2000) (Fig 1.3).

It is difficult however, from the known biochemical structure of an organism, to make a prediction regarding whether or not it will bind to MBL. Two of the most important structures that gram-negative bacteria use to assist their invasion of a host and subsequent survival are the polysaccharide capsule and endotoxin (lipopolysaccharide (LPS) or lipo-oligosaccharide (LOS)). A schematic diagram of LPS is shown in Fig 1.4 demonstrating that it consists of a lipid A moiety and an inner core which are relatively conserved between species and an outer O-antigen (α-oligosaccharide) that is very variable and contains a number of different sugars and/or sialic acid. To try and determine the relative importance of LPS structure, (specifically the presence or not of a terminal sialic acid) and the bacterial capsule in MBL's binding to an organism, experiments using a number of isogenic mutants of *Neisseria meningitides* and *Salmonella enterica* serovar Typhimurium have been conducted (Jack et al., 1998;Devyatyarova-Johnson M et al., 2000). The *N.meningitides* mutants differed in the presence or not of a capsule and/or terminal sialyation. The results obtained demonstrated that MBL bound poorly to organisms with sialylated LOS; however the absence of sialic acid allowed MBL to bind suggesting that sialic acid blocks MBL binding to its ligand (Jack et al., 2001a). In addition, the effect of the bacterial capsule was minor compared to the effect of LPS sialyation (Jack et al., 2001a) (Fig 1.5). For Salmonella the organisms with LPS truncated at various points, revealing different terminal sugars and *no* O-antigen, bound MBL but organisms with O-antigen did not bind (Devyatyarova-Johnson M et al., 2000). Binding of MBL was not able to be predicted by knowledge of just the terminal sugar and thus it is likely that sugar moieties revealed in the 3-dimesional, rather than linear, structure of LPS also play a

role in determining MBL binding. Differences in MBL binding to another component isolated from bacterial cell walls, lipoteichoic acid (LTA), have also been demonstrated and bear no obvious correlation to MBL binding to the intact organism (Polotsky et al., 1996;Jack et al., 2001a). This could suggest that MBL binds differently to bacterial components such as LTA depending on whether it interacts with it when soluble in the serum or membrane bound on the bacterial cell wall. It must also be remembered that conditions used for the binding of purified MBL to bacteria in the laboratory are significantly different from those occurring in vivo in the presence of serum which may lead to conflicting results. It is known that serum does inhibit MBL binding to some degree and that purified MBL will bind carbohydrates on sepharose beads whereas MBL in the presence of serum does not (Tan et al., 1996). Possibly serum will only inhibit low avidity interactions and therefore "low MBL binding" bacteria will become "non-binders" in the presence of serum. Another factor to consider in vivo is the presence of different MBL oligomers. It may be that only the higher oligomeric forms bind to micro-organisms with poor sugar array patterns but that all forms of MBL may bind to organisms with better fitting patterns.

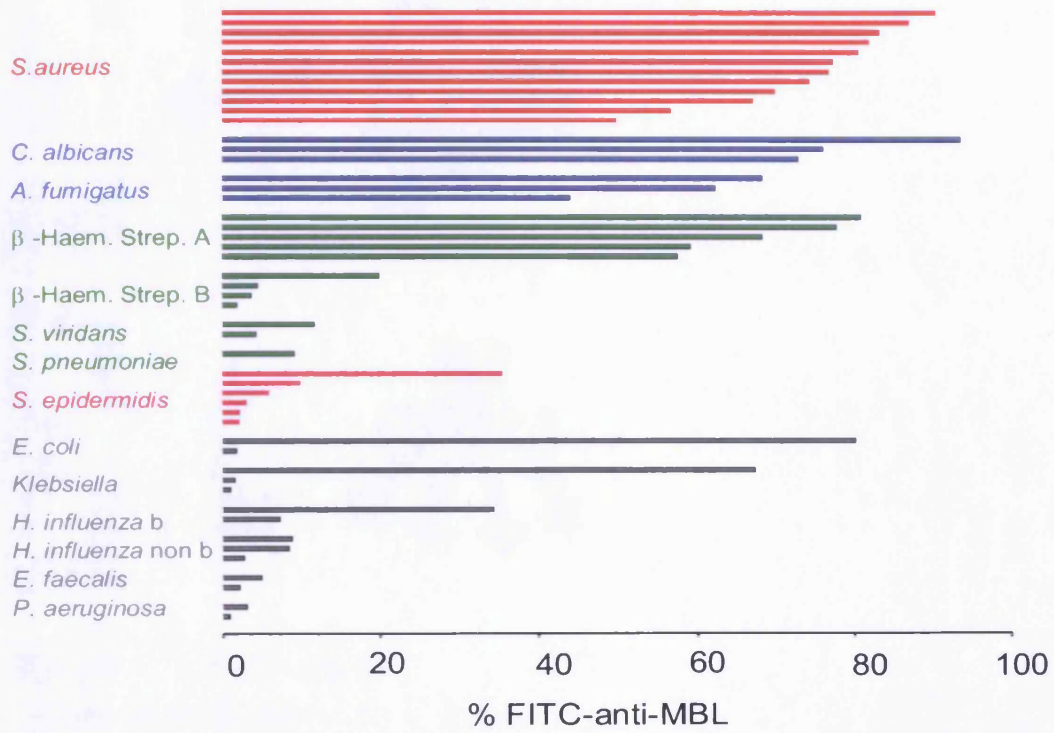


Fig 1.3 The binding of MBL to different organisms

The binding of purified MBL to pathogens was investigated by flow cytometry. Diverse *Candida species*, *Aspergillus fumigatus*, *Staphylococcus aureus*, and *beta-haemolytic group A streptococci* exhibited strong binding of MBL, whereas *Escherichia coli, Klebsiella species*, and *Haemophilus influenzae type b* were characterized by heterogeneous binding patterns. In contrast, *beta-haemolytic group B streptococci, Streptococcus pneumoniae,* and *Pseudomonas aeruginosa* showed low levels of binding (Neth et al., 2000). (Reproduced from Dr O Neth with kind permission).

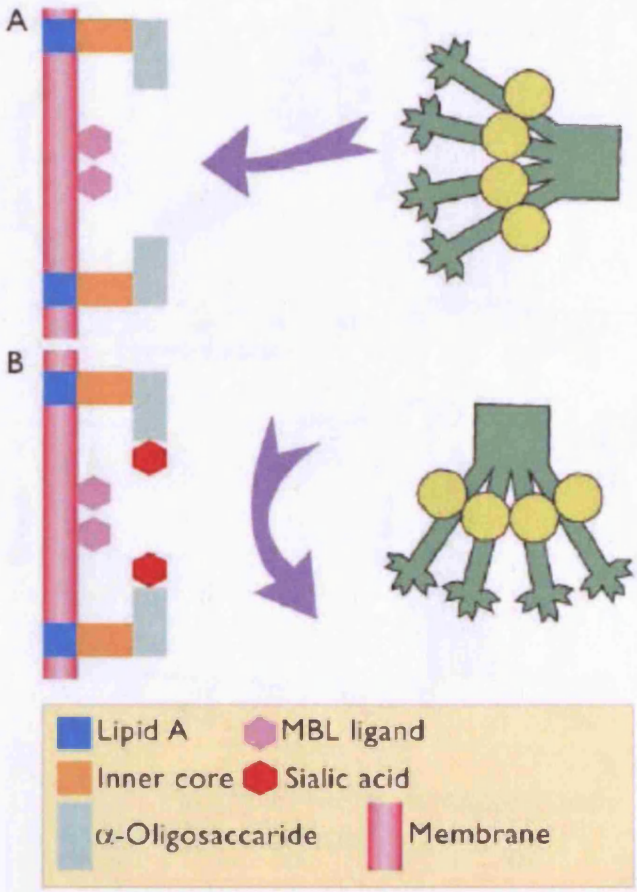


Fig 1.4 MBL binding to an organism is dependent upon lipopolysaccharide (LPS) structure.

A schematic diagram of LPS is shown demonstrating that it consists of a lipid A moiety and an inner core which are relatively conserved between species and an outer O-antigen (α-oligosaccharide) that is very variable and contains a number of different sugars and/or sialic acid. It can be seen in **A)** that the absence of sialic acid allows MBL to bind but in **B)** MBL binds poorly to organisms with sialylated LOS (kindly reproduced with permission from Prof. Klein).

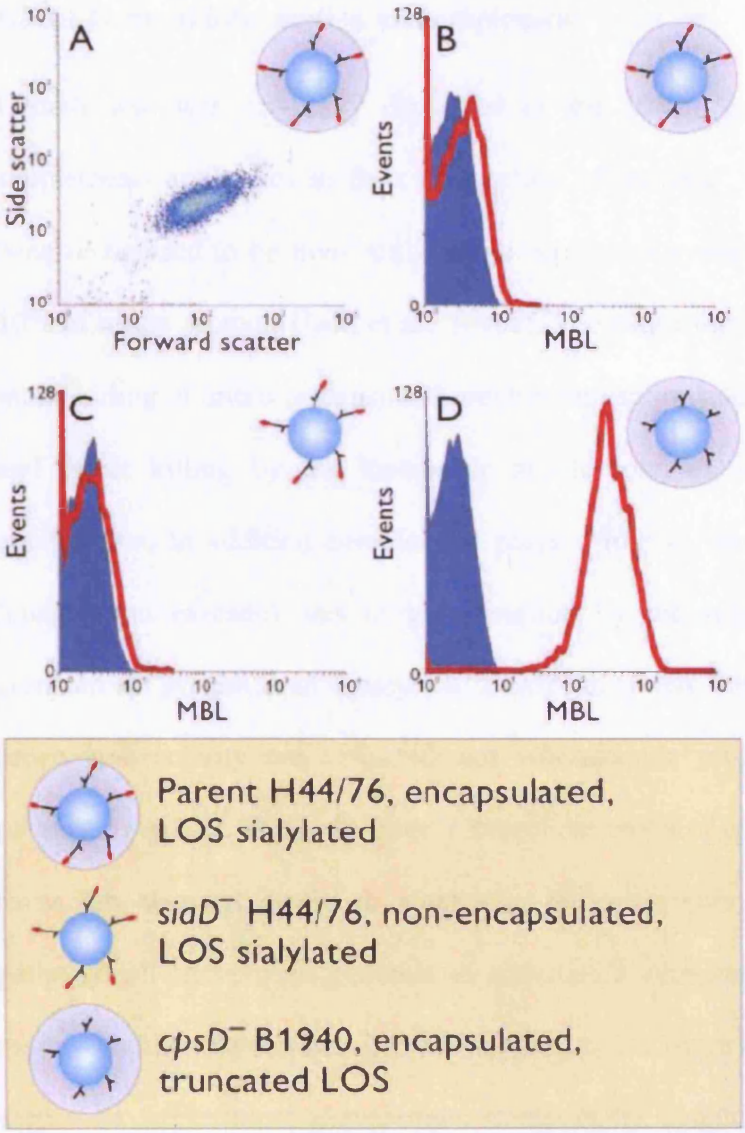


Fig 1.5 MBL binding to *N.meningitides* mutants which differ in the presence or not of a capsule and/or terminal sialyation. This flow-cytometric representation demonstrates that MBL binds poorly to organisms with sialylated LOS however the absence of sialic acid allows MBL to bind suggesting that sialic acid blocks MBL binding to its ligand. In addition the effect of the bacterial capsule is minor compared to the effect of LPS sialyation (reproduced with permission from Prof.Klein).

1.3.2 MBL and complement

1.3.2.1 General information on complement

Complement was originally described in the 1890's as "a factor in serum which supplements antibodies in their destruction of bacteria". Over 30 proteins have now been recognised to be involved in the complement system and together they make up 10% of serum proteins (Jack et al., 2001a). The major functions of complement are help in the killing of micro-organisms through opsonisation and preparation for phagocytosis and direct killing by the membrane attack complex of the terminal complement components. In addition complement plays a role in the activation of other enzymes (coagulation cascade) and in inflammation by the release of anaphylatoxins. The complement system is an example of a cascade system whereby early components with proenzyme activity are activated and subsequently cleave later components in the pathway (Fig 1.5). There are three different pathways of complement activation referred to as the classical, lectin or alternative pathways (see subsequent sections). These pathways all converge to generate an enzyme C3 convertase which activates one of the major complement proteins C3. Subsequent to this stage all pathways follow the same activation of terminal complement components resulting in the formation of the membrane attack complex. Nomenclature of this pathway is sometimes confusing as components are numbered in order of discovery and therefore are not always sequential. C3 is present in serum at approximately 1.3 mg/ml and is one of the major serum proteins. Once cleaved to C3b this can form part of the C5 convertase or alternatively act as an opsonin for the organism to which it is attached. C3b can interact with complement receptor-1 (CR1) present on phagocytes and can mediate phagocytosis in the presence of a second signal. C3b is converted to iC3b in serum by factor I. Although no longer active in complement activation, iC3b can bind with high affinity to CR3

(CD11b/Cd18) and also mediate phagocytosis at a rate that is probably more that C3b and CR1 (Ross and Densen, 1984).

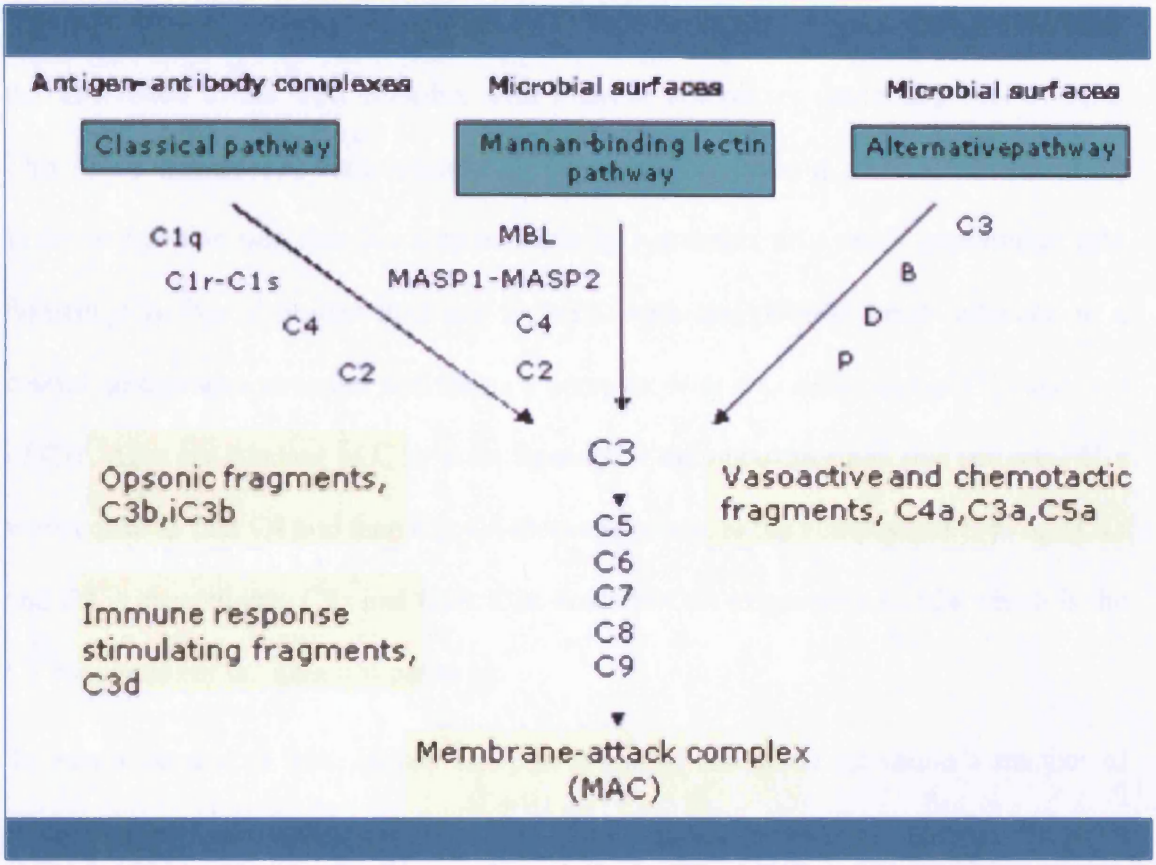


Fig 1.6 The three pathways of complement activation.

The classical pathway is activated by interaction of C1q with immune complexes and the lectin pathway by interaction of MBL with sugar arrays on the surface of micro-organisms which activates MASP's and cleaves C4 and C2. The alternative pathway is activated by microbial components. Complement components are denoted by capital letters.

1.3.2.2 The classical pathway of complement activation

This was the first pathway of complement to be described and is activated primarily by the interaction of the C1q complex with immune complexes containing IgG or IgM. This first component of complement, C1, contains three parts, q, r and s, of which C1q is the recognition unit that binds to multiple IgG domains or a single pentameric IgM domain. C1q has a similar structure to MBL with six globular heads attached to a central collagenous structure and forms a complex with two molecules of C1s and two of C1r. After the binding of C1q to its ligand C1r auto-activates and this activates C1s which cleaves first C4 and then C2. C4 cleavage produces the components C4b and C4a and C2 is cleaved into C2a and C2b. C2a binds to C4b to generate C4b2a which is the C3 convertase of the classical pathway.

To ensure control of this cascade and prevention of continued activation a number of inhibitory proteins have evolved. The first, C1 inhibitor, forms a covalent complex with activated C1r and C1s and releases C1q. The second, a serine protease Factor I, cleaves C4b at two sites rapidly stopping its biological activity. Other proteins such as the membrane bound complement receptor 1 (CR1) and C4-binding protein (C4bp) accelerate the decay of C4b2a. CR1 and other proteins such as membrane cofactor protein (MCP) are present on host cells and therefore provide protection against complement mediated host damage.

1.3.2.3 The MBL pathway of complement activation

A deficiency in complement activation was the first identifiable immune defect in humans who were subsequently shown to have MBL deficiency. Specifically, C3 was deposited in lower amounts on yeast surfaces incubated in sera from individuals that could not opsonise yeast compared to those who could (Turner et al., 1981). It was then

shown that MBL could activate the complement system when bound to mannan (Kawasaki et al., 1983;Ikeda et al., 1987). Subsequent work has defined the MBL or "lectin" pathway of complement activation in much more detail and it is now known that MBL in serum is associated with serine proteases, named MBL-associated serine proteases (MASP's), of which 4 have now been described; MASP-1, 2 and 3 and a protein with no protease activity named MAp19 (Matsushita and Fujita, 1992;Thiel et al., 1997;Dahl et al., 2001;Takahashi et al., 1999;Stover et al., 1999). The MASP proteins are not equally distributed among the different oligomers of MBL, with MAp19 and MASP-1 being associated with lower order oligomers, MASP-2 with tetramers and above and MASP-3 being found with the largest oligomers. In serum there is 20x more MASP-1 than MBL (most unbound to MBL) (Terai et al., 1997) but it seems that it is MASP-2 that is the main initiator of the complement pathway (Thiel et al., 1997) with the function of the other MASP's being uncertain. MBL (which has no catalytic function itself) binding to a target ligand induces a conformational change (cleavage of MASP polypeptide chains) causing activation of the MASP enzymes and cleavage of C4. The MBL/MASP complex then activates C2 to form C4b2a, an identical C3 convertase to that generated by the classical pathway (Fig 1.6). The exact nature of the MBL/MASP complex is unclear and the binding site of MASP to MBL is unknown but is in the collagenous region (Fig 1.2). Activated MASP-2 cleaves C4 twenty times better than activated C1s (Rossi et al., 2001) and MASP-1 can cleave complement factors C3 and C2. Mutations in the gene encoding MASP-2 have now also been identified (Stengaard-Pedersen et al., 2003;Lozano et al., 2005) and work is ongoing to determine the functional significance of these on complement activation and subsequent susceptibility to disease. In addition to this lectin pathway being activated when MBL binds to a micro-organism it can also bind to other ligands, most

importantly IgA. Here the carbohydrate recognition domain of MBL binds to polymeric but not monomeric IgA with the subsequent activation of complement which may lead to a synergistic action of MBL and IgA in antimicrobial defence (Roos et al., 2001).

The regulation of complement activation by MBL/MASP is still unclear. Alpha-2-macroglobulin (α2M) is a broad specificity protease inhibitor, abundant in serum, that was shown to bind to MASP and inhibit complement activation (Terai et al., 1995) although this was subsequently refuted (Petersen et al., 2000). In addition to binding to C1r and C1s, C1-inhibitor also binds to MASP and prevents complement activation and is probably the most important regulator of both the MBL and classical complement systems (Petersen et al., 2000).

1.3.2.3.1 Ficolins and ficolin activation of the lectin pathway

The ficolins are a group of proteins which, like MBL, bind to pathogen associated molecular patterns (PAMP's), are associated with MASP's and can activate the lectin complement pathway (Matsushita et al., 2000;Matsushita and Fujita, 2001;Matsushita et al., 2002;Lynch et al., 2004). Three ficolins are found in humans: L- and M-ficolin, which are more than 80% identical and H-ficolin (Matsushita and Fujita, 2001). Like MBL, ficolins are composed of subunits of three identical polypeptide chains with a collagenous part but in this case fibrinogen-like domains that recognise ligand (Matsushita et al., 1996). L- and H- ficolin are synthesised by hepatocytes and secreted into the circulation with M-ficolin being mainly on the surface of peripheral blood monocytes. Interestingly H-ficolin has recently been observed in bronchoalveolar fluid (Matsushita et al., 1996) and M-ficolin in type II lung epithelial cells (Liu et al., 2005) suggesting an extracirculatory role. Only one paper has reported low levels of L-ficolin being associated with recurrent infections, in this case respiratory infections in children (Atkinson et al., 2004). A study of 170 000 people only found deficiency of H-ficolin in

11 of 13 SLE patients (Inaba et al., 1990) thus it seems that ficolin deficient states are not as common or as biologically significant as MBL deficiency.

1.3.2.4 The alternative pathway of complement activation

The central component of complement, C3, is continually activated at low levels by cleavage to C3b by serum proteases or by activation to C3i. C3b is then able to form a C3 convertase with factor B in the presence of factor D. This C3 convertase, C3bBb, is stabilised by the protein properdin. The constant conversion of C3 to active forms could potentially result in host damage if it occurs on host cells. To diminish this risk a serum protein Factor H and 2 cell membrane proteins Complement receptor-1 and decay accelerating factor (DAF) promote the dissociation of the C3bBb convertase. The probability of activated C3 being deposited on the surface of a pathogen is much lower in this system compared to the more efficient classical and lectin pathways.

1.3.2.5 The terminal (lytic) pathway of complement activation

After cleavage of C3, C3b is incorporated into the C5 convertase C4b3b2a or $C3b_2Bb$ depending on the source of the C3 convertase (Fig 1.6). Cleavage of C5 yields C5a, a potent anaphylatoxin and C5b which helps in the assembly of the membrane attack complex(MAC). C5b then sequentially binds to C6, 7 and 8. C9 polymerises to form complexes of up to 18 C9 molecules which form a pore like structure and bind to C6, 7 and 8 to form the C5b6789 MAC and lysis of the target cell. Again this system has inbuilt regulatory mechanisms in the form of CR-1, DAF and two inhibitory proteins, C8 binding protein and CD59 both of which inhibit the assembly of the MAC.

1.3.3 MBL and opsonophagocytosis

MBL plays a role in opsonophagocytosis in two ways, by the direct binding to an organism i.e. acting as an opsonin and indirectly by complement activation resulting in the increased deposition of C3b and iC3b as described above. Phagocytosis of micro-organisms is known to occur by a number of mechanisms including via distinct cellular receptors for different immunoglobulin isotypes (Fc receptors) and complement coated organisms (CR 1 and CR 3). In addition the β glucan receptor on monocytes (Czop and Austen, 1985) and the mannose receptor (MR) on tissue macrophages directly recognize microbial carbohydrate configurations without the need for opsonisation (Kuhlman et al., 1989). Circulating monocytes do not express the MR but MBL seems to share similar binding characteristics to the MR and thus performs an analogous function in the serum. Using *Salmonella enterica Montevideo* organisms Kuhlman first demonstrated in 1989 that both purified and recombinant MBL increased the attachment, uptake and killing by phagocytes of organisms expressing a mannose rich polysaccharide (Kuhlman et al., 1989).

Much speculation since has centred on putative receptors responsible for mediating these effects including cC1qR/calreticulin (Malhotra et al., 1990;Ogden et al., 2001), C1qRp (Tenner et al., 1995)and CR1 (Klickstein et al., 1997;Ghiran et al., 2000). Calreticulin is expressed on the surface of many cells including neutrophils, T-lymphocytes, endothelial cells and macrophages. It does not however have a transmembrane domain and therefore after binding to the collagenous region of MBL it appears to signal through association with CD91 to initiate engulfment of the micro-organism or apoptotic cell opsonised by MBL. Antibodies to calreticulin inhibit this process (Ogden et al., 2001). C1qRp is expressed predominantly on neutrophils, monocytes, vascular endothelial cells but is absent in most tissue macrophages (Fonseca

et al., 2001). Antibodies to C1qRp inhibit both MBL and C1q mediated phagocytosis by monocytes (Tenner et al., 1995). Lastly, Complement receptor 1 (CR1) also known as CD35 has also been suggested as a cellular receptor for both MBL and C1q. It is still unclear whether MBL does infact operate as a direct opsonin for micro-organisms or whether it is enhancing other pathways such as complement or immunoglobulin receptor mediated opsonophagocytosis as depicted in Fig 1.7. The most recent data published suggests that MBL may act by stimulating organism uptake through actin cytoskeleton rearrangements in the phagocyte (Jack et al., 2005).

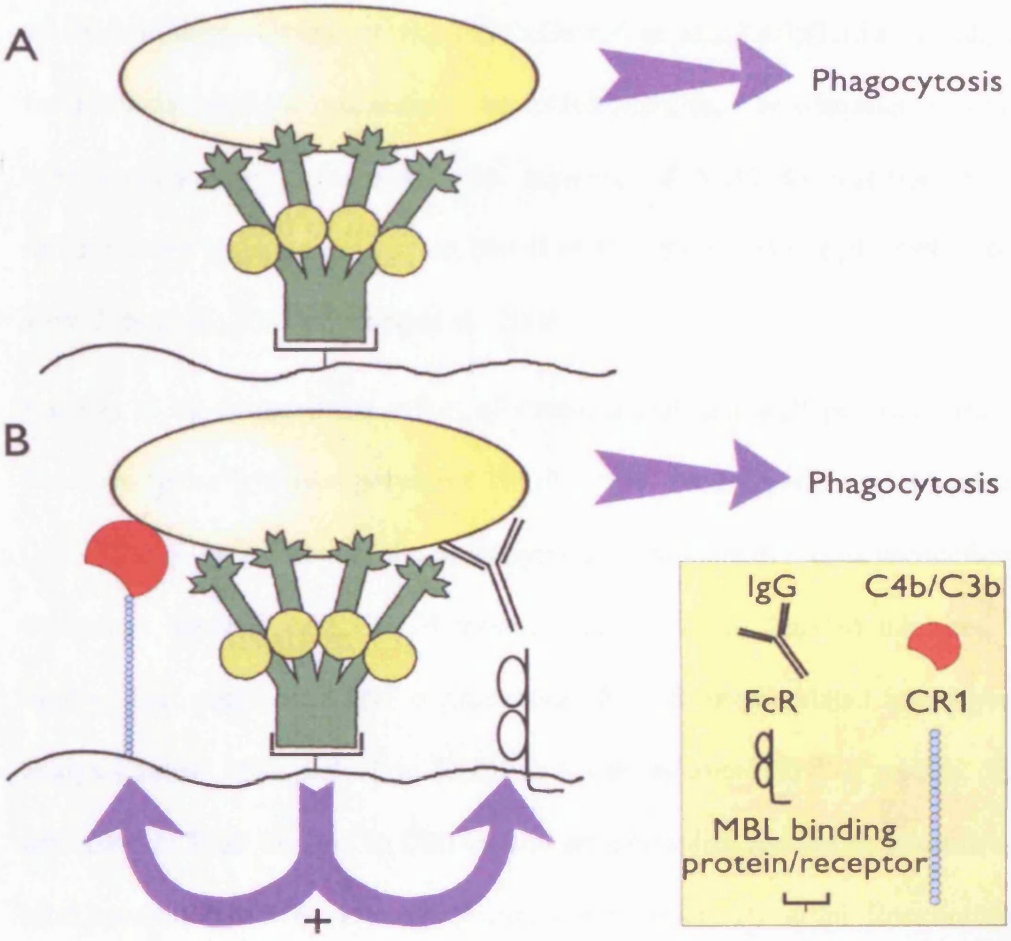


Fig 1.7 MBL and opsonophagocytosis

It is still unclear whether **(A)** MBL does infact operate as a direct opsonin for micro-organisms or whether **(B)** it is enhancing other pathways such as complement or immunoglobulin receptor mediated opsonophagocytosis. (Adapted from Jack & Klein with kind permission).

1.3.4 MBL and inflammation

There is increasing evidence, from disease association studies, that MBL also plays a role in the modulation of inflammation and thus contributes to disease severity as well as susceptibility (Garred et al., 1999b;Garred et al., 2003;Gordon et al., 2006). To further understand the mechanisms by which MBL may be operating a number of *in-vitro* studies have investigated the capacity of MBL to regulate pro and anti-inflammatory cytokine production (Soell et al., 1995;Chaka et al., 1997;Ghezzi et al., 1998;Jack et al., 2001b;Sprong et al., 2004).

Soell et al investigated the effect of streptococcal cell wall polysaccharides, formed from rhamnose glucose polymers (RGPs), on human peripheral blood monocytes (PBMC's). RGP's bound to the monocytes and stimulated TNF-α production in a dose dependent manner. Anti CD-14 monoclonal antibodies (mAbs) inhibited both RGP binding and suppressed TNF-α production from RGP-stimulated monocytes. Further analysis found MBL bound to RGPs and also inhibited TNF-α release (through the inhibition of RGP binding to CD14) with an increasing amount of inhibition occurring after incubation of MBL at concentrations of 5, 10 and 20 μg/ml. Concentrations lower than 5 μg/ml, i.e. in the clinical deficiency range were not tested. The receptor involved in the above interactions remains unknown. The C1q receptor has been suggested for mediating the binding and uptake of RGP-MBL complexes by human monocytes (Soell et al., 1995).

Chaka et al subsequently demonstrated that MBL enhanced TNF-α induction by PBMC's after stimulation with *Cryptococcus neoformans*. Interestingly this occurred at MBL concentrations of 0.31 to 1.25 μg/ml, but the effect declined at an MBL concentration of 2.5 μg/ml (Chaka et al., 1997).

Jack et al demonstrated that the addition of MBL to the ex-vivo blood of MBL-deficient donors, after bacterial stimulation with *N.meningitidis,* influenced the production of monocyte-derived inflammatory cytokines in a complex manner. Specifically, high concentrations of MBL (>6000ng/ml), such as seen in an acute phase response, decreased the production of interleukin-6 (IL-6), interleukin -1β (IL-1β) and tumour necrosis factor-α (TNF-α) by monocytes, whereas lower concentrations (<1000-2000ng/ml), such as seen in MBL homo or heterozygotes, enhanced the production of IL-6 and IL-1β.

Sprong et al used a different model of stimulating human peripheral blood mononuclear cells (PBMC's) with lipopolysaccharide (LPS) + or LPS- meningococci in a system with no exogenous complement. Here only lower concentrations of MBL were used (0.75-2500 ng/mL), different cytokines were assessed (IL-1β, IL- 6, IL-10, TNF-α and IFN-γ) and different results were obtained. The main findings were that MBL (<2500 ng/mL) significantly augmented IL-1β production after stimulation with both LPS+ and LPS- meningococci in a dose dependent fashion and also enhanced IL-10 production by LPS- meningococci. In contrast the production of IL- 6, TNF-α and IFN-γ were unaffected.

1.3.5 MBL and apoptosis

The removal of apoptotic cells is essential for the maintenance of a number of homeostatic mechanisms in humans, including maintenance of the immune system, protection against neoplasia and resolution of inflammation. It has been demonstrated that mannose binding lectin (like C1q) can bind to apoptotic cells and enhance phagocytosis by macrophages. This process was shown to involve MBL binding, at the

collagenous region, to the cell surface protein calreticulin (also known as the cC1qR). As cC1qR has no transmembrane domain it in turn is bound to the endocytic receptor protein CD91 (also known as the alpha-2-macroglobulin receptor) on the macrophage surface and initiates engulfment by macropinocytosis (Ogden et al., 2001). Further work has confirmed that MBL binds apoptotic cells via the CRD and that the stage of apoptosis is important. By flow cytometry and fluorescence microscopy, MBL can be seen to bind specifically to late apoptotic cells, apoptotic blebs and necrotic cells but not to early apoptotic cells and binding did not lead to activation of the lectin pathway. MBL may therefore facilitate the uptake of apoptotic cells by macrophages and promote the non inflammatory process of programmed cell death (Nauta et al., 2004). This also may contribute to the underlying mechanism by which lack of MBL is associated, in clinical studies, with increased inflammation in certain situations (described below).

1.4 MBL genetics

1.4.1 MBL gene structure

There are two human *MBL* genes of which *MBL-1* is a pseudogene and only *MBL-2* encodes the protein product, MBL. *MBL-2* has been mapped to the long arm of chromosome 10 (q11.2-q21) (Sastry et al., 1989) which is close to the genes for the other collectins; SP-A at 10q21-24 (Bruns et al., 1987) and SP-D at 10q 22.2-23.1 (Crouch et al., 1993). The gene consists of four exons with an upstream promoter region. Exon 1 encodes the amino terminal section comprising the cysteine rich region and the first part of the collagenous region. Exon 2 encodes the rest of the collagenous region and exon 3 the "neck" region. The fourth and largest exon encodes the carbohydrate recognition domain (see previous Fig 1.2).

1.4.2 Regulation MBL gene transcription

Examination of the nucleotide sequence upstream of the *MBL-2* gene (promoter region) revealed several regulatory elements including several features that were characteristic of acute phase protein genes such as a heat shock consensus element at -592 base pair (bp) and a glucocorticoid-responsive element that may enhance MBL transcription (Taylor et al., 1989). MBL was subsequently demonstrated to be a modest acute phase protein with serum levels increasing between about 1.5 - 3 times the "resting" level after surgery or during an acute infection (Thiel et al., 1992). Gene transcription is also enhanced by interleukin-6 (IL-6) and dexamethasone in human hepatoma cell lines (Arai et al., 1993).

1.4.3 Genetic control of MBL serum concentration

MBL serum concentrations differ markedly between individuals and between ethnic populations with the median serum concentration in a well Caucasian population being approximately 995 ng/ml with an interquartile range of 642-1410 ng/ml. Some variation is expected as MBL is an acute phase protein; however the situation is more complex than this as levels ranging from undetectable to over 6 000 ng/ml have been demonstrated (Garred et al., 1999b). This range is principally due to mutations in the structural exon 1 part of the MBL gene although polymorphisms in the promoter region also play an important role.

1.4.4 MBL structural gene mutations

Three mutations, all in exon 1, resulting in an amino acid substitution in the MBL polypeptide chain have been described. These occur at codons 52, 54 and 57 and are known as the D, B and C variants respectively as opposed to A which indicates the wild type (normal) gene. The first identified was a G to A base change in codon 54 (GGC to

GAC) resulting in glycine to aspartic acid substitution in the collagen domain (Sumiya et al., 1991) and occurs in approximately 26% of Caucasians (Mead et al., 1997). The second mutation described at codon 57 (GGA to GAA) was initially identified in a Gambian population, with a prevalence of 58% and results in a glycine to glutamic acid substitution (Lipscombe et al., 1992b). The third mutation was identified in 1994 at codon 52 resulting in a C to T base change (CGT to TGT) which translates into an arginine to cysteine substitution. This allele is only present in approximately 5% of both Caucasian and African populations (Madsen et al., 1994). Both the 54 and 57 variants result in very low serum levels caused by the replacement of a small glycine residue by a large aspartic or glutamic acid leading to disruption to the collagen like domain of MBL. In addition, the disulphide bonds of the oligomers are disrupted resulting in the formation of unusual two and four polypeptide chains (Wallis and Cheng, 1999). The codon 52 mutation may not disrupt the triple helix itself but may just produce extra disulphide bonds which prevent the assembly of higher oligomers and thus serum levels in individuals with this mutation are not as low as with the 54 and 57 mutation (Fig 1.8). Thus MBL deficiency may not in-fact represent a complete lack of MBL in the serum but represents the presence of circulating monomeric or oligomeric mutant MBL protein with impaired function (Terai et al., 2003). Different detection methods pick up different levels of MBL (see Methods section 2.3.1).

These three structural mutations show marked difference in prevalence amongst different populations across the globe. The codon 54 mutation is present in Eurasian populations whereas the codon 57 mutation is found almost exclusively in sub-Saharan Africans. The codon 52 mutation occurs at low levels worldwide (Table 1.1). As the mutations seem to be split mainly along African and non-African boundaries it has been suggested that these mutations occurred independently after the divergence of these

populations approximately 150 000 years ago (Lipscombe et al., 1992b). That these mutations with a similar phenotypic effect have occurred and persisted at such high gene frequencies in such diverse populations is suggestive of a biological advantage in some situations (see section 1.6.4).

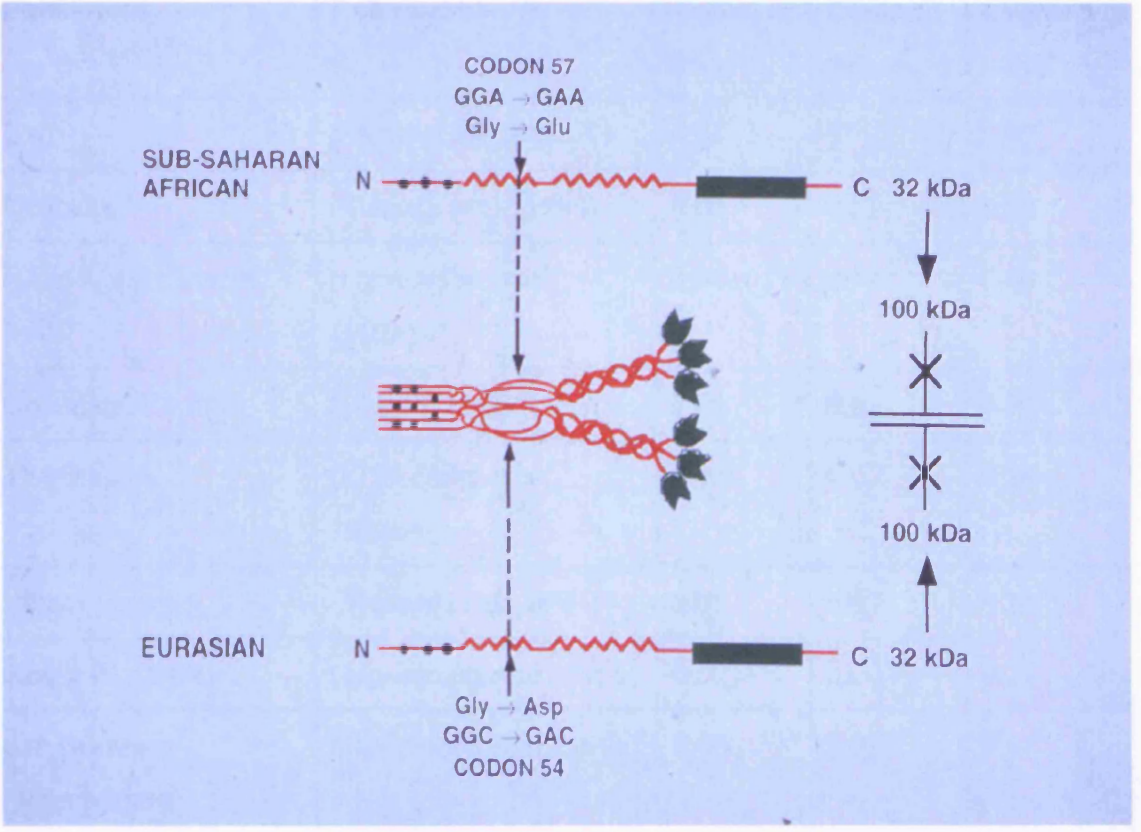


Fig 1.8 The effect of MBL genetic polymorphism on the translated protein.

The 54 and 57 variants cause the replacement of a small glycine residue by a large aspartic or glutamic acid leading to disruption to the collagen like domain of MBL and failure to form higher order oligomers.

		Observed allele frequency		
Population	**Reference**	**Codon 52**	**Codon 54**	**Codon 57**
UK	(Mead et al., 1997)	0.07	0.14	0.02
Denmark	(Madsen et al., 1994)	0.05	0.13	0.02
Hong Kong Chinese	(Lipscombe et al., 1992a)	0.01	0.11	0.00
Greenland Eskimos	(Madsen et al., 1994)	0.00	0.13	0.00
The Gambia	(Lipscombe et al., 1992b)	0.02	0.00	0.29
Kenya	(Madsen et al., 1994)	0.05	0.03	0.23
Xhosa (S.Africa)	(Lipscombe et al., 1996)	0.00	0.00	0.27
San Bushmen (Namibia)	(Lipscombe et al., 1996)	0.00	0.03	0.07
Papua New Guinea	(Lipscombe et al., 1996)	0.00	0.07	0.00
Vanuatu (SW Pacific)	(Lipscombe et al., 1996)	0.00	0.01	0.00
Quechua Indians (Peru)	(Garred et al., 2006)	0.00	0.80	0.00

Table 1.1 Frequencies of mutant alleles of the *MBL-2* gene in various populations. This shows the marked geographical and ethnically related variation in the prevalence of the *MBL-2* gene mutations. In some populations such as Vanuatu, mutations are extremely rare whilst in others such as The Gambia and the Quechua Indians in Peru mutations are extremely common suggesting some kind of selective advantage.

1.4.5 Polymorphisms of the MBL promoter region

There are several polymorphisms within the promoter region of the *MBL-2* gene which affect serum MBL levels. These are as follows: X/Y (C/G) polymorphism at -221bp, the H/L (G/C) at -550bp and the P/Q polymorphisms at -427 (A/C), -349 (A/G), -336 (A/G), deletion -329 to -324, -70 (C/T) and +4 (C/T) (Madsen et al., 1995;Madsen et al., 1998a). Four promoter haplotypes are commonly seen, namely LXP, LYP, LYQ and HYP of which HYP is the highest MBL producing haplotype, followed by LYQ and then LYP. The LXP haplotype produces the lowest MBL serum levels. The combined effect of the structural gene mutations and promoter region polymorphisms affect MBL serum concentrations with the influence of the X/Y polymorphism being the most profound after the effect of the structural mutation. Due to significant linkage disequilibrium between the exon 1 mutations and promoter polymorphisms the following haplotypes are linked: LYP and codon 54 mutation, HYP and codon 52, LYQ and codon 57 (Madsen et al., 1995;Madsen et al., 1996). A study of Danish Caucasians with the normal exon 1 genotype (A/A) demonstrated that those with the promoter HYP i.e. HYPA/HYPA haplotype were found to have a serum MBL level six times higher than those with the haplotype LXPA/LXPA (means of 2279ng/μl and 346 ng/μl respectively) (Madsen et al., 1995). As the X/Y promoter polymorphism has the greatest effect on serum level this is the one that is most commonly determined in MBL gene-disease association studies.

1.5 MBL levels in serum and other body fluids

MBL is primarily a serum protein although it has been identified in pleural and ascitic fluid, in patients with disease, at mean levels of 690 and 420 ng/ml respectively. Much lower levels of 5 and 0.5 ng/ml were found in CSF and urine respectively (Fig 1.9) (Terai et al., 1993). At the commencement of this PhD no one had demonstrated MBL in airway secretions such as bronchoalveolar lavage fluid.

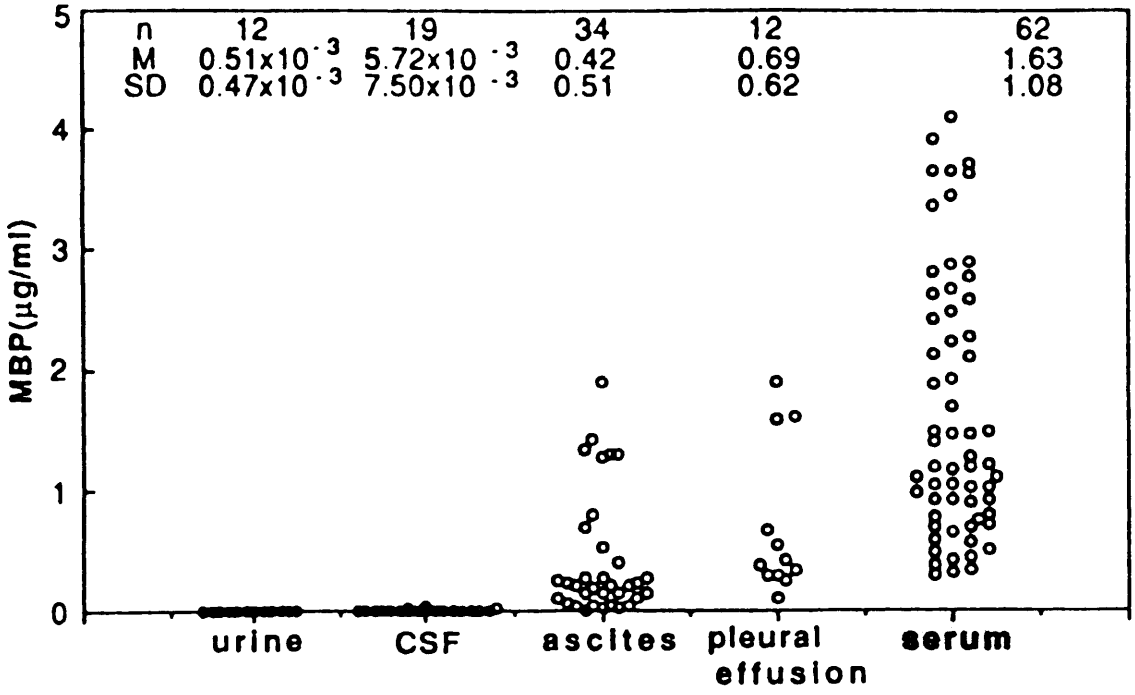


Fig 1.9 MBL levels in serum and other body fluids.

This scattergram, taken from Terai 1993, demonstrates the considerable difference in MBL levels in different body fluids. At the commencement of this thesis no studies had looked for MBL at the airway surface in humans. (Mean MBL levels shown here are as follows: Serum 1600 ng/ml, pleural effusion 690 ng/ml, ascites 420 ng/ml, CSF 5 ng/ml urine 0.5 ng/ml).

1.6 MBL disease associations

1.6.1 MBL and susceptibility to infectious diseases

Over the last two decades the clinical importance of MBL has been increasingly highlighted by a number of studies demonstrating the role of MBL deficiency in *susceptibility* to infectious diseases. It now seems evident that MBL also affects the *severity* of the clinical disease (see section 1.6.3).

The first description of low levels of MBL and frequent infections was described in 1989 (Super et al., 1989) followed in 1991 by a description of 4 family members with frequent unexplained infections who were all homozygous for the MBL codon 54 mutation (Sumiya et al., 1991). In a large cohort of consecutive children admitted to hospital, categorised as having infection or not, the prevalence of MBL gene mutations in those with infection was almost twice that of children admitted without infection. Here both homozygosity and heterozygosity for an MBL mutation was associated with an increased susceptibility to infection (Summerfield et al., 1997). Further studies have found an association between MBL deficiency and meningococcal disease (Hibberd et al., 1999), Mollaret's meningitis (recurrent aseptic meningitis) (Tang et al., 2000), unusual and severe infections in adults (Summerfield et al., 1995), susceptibility to mycoplasma infection in patients with primary antibody deficiencies (Hamvas et al., 2005) and most recently in the susceptibility to, but not severity from, the newly described severe acute respiratory syndrome (SARS) caused by the novel coronavirus (SARS-CoV) (Ip et al., 2005).

1.6.1.1 MBL and respiratory infections

Childhood respiratory infections result in significant morbidity worldwide and bacterial respiratory infections are a leading cause of death in the developing world. MBL variant

alleles have been associated with a number of respiratory tract infections, although there are now some conflicting data published.

Koch's group followed up 252 children, under the age of two years, weekly for 2 years for morbidity surveillance and demonstrated a 2 fold increased relative risk of acute respiratory infection in those MBL insufficient children compared to MBL sufficient children (Koch et al., 2001). This risk was mainly in children aged 6-17 months and thus could suggest that MBL plays an important role in host defence during the period of life that maternal antibody is waning and the adaptive response is not yet mature. Symptoms and signs detected were broad, ranging from purulent rhinitis to bronchiolitis and pneumonia. Causative organisms were not looked for. MBL is known not to bind to respiratory syncytial virus (RSV) in vitro and a study looking at 79 infants less than 6 months of age found no association between MBL variant alleles and RSV incidence or disease severity (Kristensen et al., 2003).

Regarding pneumococcal disease, a study of 229 UK patients aged 0-94 years with invasive pneumococcal disease demonstrated an overrepresentation of MBL homozygotes (but not heterozygotes), in patients compared to controls (odds ratio 2.59). This finding was confirmed in a second cohort of patients in a similar geographical area (Roy et al., 2002). In a smaller cohort of Danish adults however no association was seen (Kronborg et al., 2002)

1.6.1.2 MBL and HIV

Death from HIV/AIDS has now overtaken both tuberculosis and malaria as the most common cause of death worldwide from an infectious disease. In 2001, 3.1 million deaths were estimated to be from HIV and 42 million people were thought to be HIV infected, mainly in Sub-Saharan Africa (Bates et al., 2004). Marked inter-individual

differences in disease susceptibility and disease phenotype have been noted by clinicians for many years. The first study to determine the possible role of MBL was that by Garred in which a significantly higher frequency of people homozygous for an MBL mutation were found in a cohort infected with HIV compared to a matched control group with the same risk taking behaviour (Garred et al., 1997a). This was subsequently confirmed in other studies (Garred et al., 1997b;Nielsen et al., 1995) but most recently refuted in a spanish cohort (Garcia-Laorden et al., 2006). A number of groups have now reported an increase in mother to child transmission of HIV in the presence of MBL polymorphisms (Boniotto et al., 2000;Boniotto et al., 2003;Crovella et al., 2005). Regarding disease progression, MBL variant alleles have been associated with a *slower* progression to an AIDS diagnosis and death in adults (Maas et al., 1998) but most recently MBL mutations were found to be *under*-represented in HIV infected children who were long term non-progressors (Dzwonek et al., 2006). Differences may be due to different geographical cohorts or more likely differences in the ages of the individuals, children usually having been infected since birth thus in a developing immune system and the adults at a much later date. MBL has been shown to bind to the glycosylated glycoprotein gp 120 in the HIV viral coat and possibly inhibits HIV infection in vitro (Ezekowitz et al., 1989).

1.6.1.3 MBL and infections in the immunocompromised host

Individuals with primary or secondary immunodeficiencies may rely on components of the innate immune system for defence against infection more than the immunocompetent host. This has already been discussed with regard to susceptibility to mycoplasma infection in patients with primary antibody deficiencies (Hamvas et al., 2005). A time when the role of MBL may also be crucial is during the period of neutropenia that is seen in patients undergoing chemotherapy for malignancy. Two

initial studies in 2001 demonstrated that this is indeed the case. Neth el al found in 100 children undergoing chemotherapy that those with MBL variant alleles spent twice as many days in hospital with febrile neutropenia compared to wild type individuals (Neth et al., 2001). In the same issue of The Lancet, a second study was reported of 54 adults undergoing chemotherapy in whom those who developed significant infections had significantly lower levels of MBL than those without significant infections (Peterslund et al., 2001). A more recent study, however, reported no effect of MBL on infective complications or mortality in adult patients with acute myeloid leukaemia (Bergmann et al., 2003).

1.6.2 MBL and disease *severity*

A number of studies have now highlighted the role that MBL deficiency plays in disease severity as well as susceptibility. These include cystic fibrosis (see section 1.7.2), sepsis (see section 1.8), HIV (see section 1.6.1.2), hepatitis (Yuen et al., 1999), recurrent miscarriages (Kilpatrick et al., 1995), Kawasaki's disease (Biezeveld et al., 2006), myocardial infarcts in patients with severe atherosclerosis (Madsen et al., 1998b) to mention just a few. Further discussion of this is found in chapters 3, 6 and the final discussion.

1.6.3 MBL and susceptibility to autoimmune diseases

The aetiology of autoimmune disease is still unclear but is thought to occur in "genetically susceptible" individuals with a possible infective trigger. The known association with deficiency of the complement component C1q and immune complex disease has prompted clinical association studies with MBL. A number of studies and meta-analyses have now demonstrated that indeed MBL deficiency and variant alleles are associated with both susceptibility to and complications from SLE (Garred et al.,

1999a;Ohlenschlaeger et al., 2004;Lee et al., 2005) and rheumatoid arthritis (Graudal et al., 1998). The mechanism is thought to be lack of removal of immune complexes by the classical complement pathway (C1q) and MBL (directly or indirectly).

1.6.4 Advantages of MBL deficiency

The high frequency of variant alleles in some populations (see Table 1.1) suggests that MBL deficiency may confer some biological advantage in certain communities, like the carriage of sickle cell trait and protection from malaria (Allison, 1954). One hypothesis is that MBL deficiency may reduce activation of the complement system and reduce host damage due to subsequent release of inflammatory mediators (Lipscombe et al., 1992b). This is now thought *not* to be the whole case as MBL deficiency has infact been associated with pro-inflammatory states (see chapter 6). Secondly, and increasingly likely, is the theory that MBL deficiency may *contribute* to host defence against certain intracellular organisms. Bacteria such as leishmania, mycobacteria and legionella use C3 opsonisation and C3 receptors on monocytes/macrophages to gain entry into the host cell and thus any reduction of complement activation may *reduce* the risk of infection with these intracellular organisms. Evidence in support of this is found in clinical studies showing significantly higher MBL levels in patients a) infected with *Mycobacterium leprae* than in healthy controls (Garred et al., 1994)and b) with visceral leishmaniasis than healthy controls (Santos et al., 2001). The presence of the MBL C allele has also been shown to be protective against pulmonary tuberculosis in Gambian adults (Bellamy et al., 1998).

1.7 Cystic fibrosis

1.7.1 Cystic fibrosis background

Cystic fibrosis is both the most common autosomal recessive disease and the most frequent lethal genetic disorder of Caucasians worldwide. 1:25 Caucasians are carriers of the defective gene resulting in a CF prevalence of 1:2500 newborns, with more than 6, 000 affected patients in the UK.

The disease is due to abnormalities in the CF transmembrane regulator (CFTR) protein, a cAMP-activated chloride channel located in the apical membrane of most secretory cells. CFTR is primarily a chloride-ion channel but it also inhibits the epithelial sodium channel. CF respiratory epithelia (and pancreas, gut, liver and reproductive tract epithelia) therefore fail to secrete chloride ions and hyperabsorb sodium ions. Water is absorbed with the sodium, resulting in dehydrated, viscous secretions with secondary end organ damage, most prominent in the lung where inspissated mucous impairs mucociliary clearance resulting in both infection and inflammation. In the sweat glands there is a failure of absorption of both chloride and sodium ions resulting in high sweat electrolytes.

1.7.2 CF genetics

The gene encoding CFTR protein is located on chromosome 7 and was first identified in 1989 (Riordan et al., 1989). Over 1,000 mutations have been identified to date. The commonest mutation is a deletion of a phenylalanine residue at codon 508 (*ΔF508*), found on approximately 67% of CF chromosomes. Most clinical laboratories screen for 31 of the commonest mutations which detects more than 90% of affected Caucasians. The CF genotype is not well correlated with phenotype apart from in pancreatic disease where, for example, the genotype R117H/ ΔF508 is associated with pancreatic

sufficiency and older age of diagnosis compared with ΔF508 homozygous patients (CF consortium, 1993). Screening for CF was initiated nationwide in the UK in 2006.

1.7.3 CF presentation

Clinically the disease causes a wide range of signs and symptoms (see below) and may now also be diagnosed by the neonatal screening programme, by the screening of a relative of a patient or by antenatal testing in a family with an affected child. The commonest presentation in a neonate is with meconium ileus and bowel obstruction. In older children it is with failure to thrive and/or recurrent respiratory infections.

- **Pulmonary manifestations**

Persistent lower airway infection and inflammation with resultant bronchiectasis is the main cause of morbidity and mortality in CF. Infection is most commonly with the pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa* (80% chronically infected by adolescence). Others such as *Burkholderia cepacia complex*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia* and atypical mycobacteria also have a propensity for the milieu of the CF lung. This manifests clinically as recurrent chest infections, persistent sputum production, reduced exercise tolerance and reduced lung function.

Whether inflammation follows or precedes infection is a matter of recent controversy (Rao and Grigg, 2006). A decade ago Armstrong's group compared bronchoalveolar lavage (BAL) fluid from 46 infants under 6 months old, identified from a neonatal screening programme, with 13 controls. Patients were divided into those who had never had an infection, those with a definite current infection and those with a possible infection. BAL's were analysed for cell counts, interleukin-8 (IL-8) and neutrophil elastase. Those patients without infection had BAL profiles similar to the control

patients and those with infection had evidence of airway inflammation thus suggesting that inflammation *follows* infection (Armstrong et al., 1997). There is good evidence to support the hypothesis that lung damage is due to mediators released by neutrophils, e.g. neutrophil elastase, that have migrated to the lung in response to chronic infection (Sagel et al., 2001) and that reducing infection attenuates lung inflammation (Ordonez et al., 2003). However, more recently the lungs of CF and non-CF fetuses were examined and an increased number of macrophages (a major source of neutrophil chemoattractants) were seen prior to birth (Hubeau et al., 2001). Another study found an increased number of neutrophils in the BAL's of young CF infants without clinical or microbiological evidence of infection (Rosenfeld et al., 2001). In addition, CF monocytes cultured in heterologous serum, produced more tumour necrosis factor-alpha (TNF-α) than control monocytes and fresh cultured CF bronchial epithelial cells produced more interleukin-6 (IL-6) and IL-8 and much less IL-10 than non-CF cells. Taken together this suggests that some CF cells may be "primed" to be in a pro-inflammatory state compared to non CF cells even prior to infection (Pfeffer et al., 1993).

- **Pancreas**

Exocrine pancreatic enzyme insufficiency occurs presenting with steatorrheoa and failure to thrive. This is treated by enzyme supplementation (e.g. Creon) and fat soluble vitamin supplementation. Endocrine problems i.e. diabetes occurs sometimes in older children (8-15%).

- **Gastrointestinal tract**

Distal intestinal obstruction syndrome (DIOS) and hepatic cirrhosis may occur.

- **Upper respiratory tract**

Up to 30% of CF patients have nasal polyps which may be treated with topical steroids or surgery. Sinusitis is common, due to obstruction and caused by the same organisms as found in the lung.

- **Infertility**

99% of males are infertile with women having sub-fertility, although many have now successfully had children.

1.7.4 CF diagnosis

The "gold standard" is a sweat test where a value of chloride ions (best) or sodium ions of >60mEq/l are diagnostic of CF in a patient with a typical history (there are also a number of conditions that can give a false positive result). A high level of immunoreactive trypsin (IRT) in the serum in the first 2-3 weeks of life is highly suggestive of CF and currently used as the CF screening tool (blocked pancreatic ducts prevent trypsinogen from reaching the intestines resulting in a build up of the protein in the blood). Gene mutations can then be looked for.

1.7.5 CF treatment

The mainstay of therapy remains chest physiotherapy twice daily whilst clinically well in addition to during an exacerbation. Prophylactic oral anti-staphylococcal antibiotics are used from diagnosis in those under 4 years of age in the UK and most of Europe and respiratory infections are treated aggressively, with oral, intravenous and inhaled antibiotics, depending on the nature of the organism. Aerolised mucolytics (eg. rhDNase) are used to decrease sputum viscosity and aid expectoration. Nutrition is optimised and approximately 85% of children need pancreatic and vitamin supplementation. Psychosocial support for children and families with CF is also offered.

Recent attention has focused on mechanisms to reduce inflammation in CF lung disease including the use of macrolides, steroids and non-steroidal anti-inflammatory drugs (Jaffe and Bush, 2001). Anti-inflammatory treatments may of course abolish the useful effects of inflammation if used incorrectly. This has been highlighted by the premature stopping of a clinical trial of a leukotriene B4 receptor antagonist in CF patients due to an increase in the number of pulmonary infective exacerbations in the treatment group (Rao and Grigg, 2006). Further work which may shed light on mechanisms involved in the modulation of inflammation in the CF lung is one of the aims of this thesis.

1.7.6 Genetic modifiers of cystic fibrosis (including MBL)

Poor correlation of CF genotype with phenotype has led to large body of work looking at the possibility of secondary genes that may modify CF disease progression. Discordant phenotypes amongst siblings with the same *CFTR* mutation brought up in the same household with the same environmental influences has further highlighted the possibility of modifier genes (Salvatore et al., 2002) (see chapter 3 introduction).

The link between MBL deficiency and CF was first described in 1999 and is also described in detail in chapter 3.

1.8 Sepsis and the systemic inflammatory response syndrome (SIRS)

1.8.1 MBL and sepsis

At the start of this project no work had been published, in adults or children, looking at the effect of MBL in sepsis or the systemic inflammatory response syndrome (SIRS). This topic is discussed in detail in chapter 6.

1.9 Aims of this Thesis:

The aims of this project were to investigate the role of MBL in two diseases where both infection and inflammation are implicated in the pathophysiology.

The first disease, cystic fibrosis (described in section 1.7), is a multisystem chronic disease characterised by a propensity to specific respiratory infections with a marked inflammatory response. The second disease is an acute, multisystem, inflammatory response to an infectious or non infectious insult, the *systemic inflammatory response syndrome* (SIRS) (see chapters 6).

It was hoped that by undertaking studies in these two populations that the role that MBL plays in both infection and inflammation may be teased out and the hypothesis that MBL plays a role in modulating the inflammatory response, as well as in susceptibility to infection, may be tested in primarily a clinical setting.

The specific aims of this thesis are described in each chapter.

CHAPTER 2

Materials and General Methods

2.1 Reagents and materials

2.1.1 Standard media and solutions

Name	Company	Product code
Agarose, electrophoresis grade	Gibco™ Invitrogen Corporation	15510-019
Ammonium persulphate (APS), $(NH_4)_2S_2O_8$	Sigma	A9164
AmpliTaq Gold DNA polymerase	Perwin elmer Applied Biosystems	N808-0241
Biotinamidocaproate N-hydroxysuccinamide ester	Sigma	B2643
Bovine serum albumin, low endotoxin	Sigma	A2934-100G
Brefeldin A (10μg/mL)	Sigma	
Bromphenol blue (3',3",5',5"tetra-bromophenolsulfonaphthalein), sodium salt, $C_{19}H_9Br_4O_5SNa$	Bio-Rad	161-0404
Calcium chloride (dehydrate), $CaCl_2x2H_2O$	Sigma	C3881
Cellfix (1% formaldehyde, 0.1% sodium azide)	Becton Dickinson	340181
Citric acid, C (OH) CO2H (CH_2CO_2H)	BDH	10081
Dimethyl sulphoxide (DMSO)	BDH	103232
DNA ladder, 1 Kb	Gibco™ Invitrogen Corporation	15615-024

2'-Deoxynucleoside 5'-triphosphate (dNTP) set 100mM	Invitrogen stores	10297018
Ethanol, C_2H_5OH	Hayman	UN1170
Ethenediaminetetraacetic acid, di-sodium salt (EDTA), $C_{10}H_{14}N_2O_8Na_2x2H_2O$	Sigma	E5143
Ethidium bromide tablets, $C_{21}H_{20}N_3Br$	Sigma	E2515
FACSflow	Becton Dickinson	342003
FACS lysing buffer	Becton Dickinson	349202
FACS fix	Becton Dickinson	
Film, scientific X-OMAT AR	Kodak	1651454
Fluoroscein isothiocyanate FITC	Sigma	F7250
Galactose D-(+)	Sigma	G0750
Hank's balanced salt solution with and without Ca^{2+} and Mg^{2+} salts	Gibco™ Invitrogen Corporation	24020-091 14025-050
Heparin sodium (10 IU/mL)	CP Pharmaceuticals	PL4543/0208
Hydrogen peroxide (30% w/w), H_2O_2	Sigma	H1009
100bp ladder	Invitrogen stores	15628019
Leukoperm	Serotec Ltd	
L-Glutamine 200mM (100x), liquid	Gibco™ Invitrogen Corporation	25030-024
Lipopolysaccharide, E.coli O111B: 4, gel purified	Sigma	L4391
Loading dye, 6x	Sigma	G7654
Magnesium chloride (hexahydrate) $MgCl_2.6H_2O$	Sigma	M8266-100G
Mannose D-(+)	Sigma	M6020
2-mercaptoethanol	Sigma	M6250
MilliQ water from Millipore Q plus purification system	Millipore	
Paraformaldehyde $(CH_2O)_n$	Sigma	P6148
PBS tablets	Oxoid	BR0014g
PBS (Dulbecco solution)	Gibco™ Invitrogen Corporation	14190-094

Protogel, containing acrylamide (30% w/v) and bisacrylamide (0.8% w/v)	National Diagnostics	EC-890
QIAMP DNA extraction kit (250)	Qiagen	51106
RPMI 1640 basal medium	Gibco™ Invitrogen Corporation	22511-026
RPMI 1640 medium with 25mM HEPES and 10mM L-glutamine	Gibco™ Invitrogen Corporation	52400-041
RPMI 1640 medium without phenol red	Gibco™ Invitrogen Corporation	32404-014
Skimmed milk	Marvel	
Sodium azide, NaN_3	BDH	10369
Sodium bicarbonate, $NaHCO_3$	BDH	10247 4V
di-Sodium borate, $Na_2B_4O_7x10H_2O$	BDH	10267 4E
Sodium carbonate, Na_2CO_3	BDH	10240
Sodium chloride, NaCl	BDH	10241
SDS (sodium dodecyl sulphate [Lauryl sulphate])	Sigma	L4390
SDS-PAGE standards (biotinylated markers)	Bio-Rad	161-0311
SDS-PAGE standards (Rainbow markers)	Amersham Pharmacia	RPN 756
Sodium hydrogen carbonate, $NaHCO_3$	BDH	102474V
di-sodium hydrogen orthophosphate, Na_2HPO_4	Sigma	S0876-500G
Sodium hydroxide, NaOH	BDH	102524X
Streptavidin Horse Radish Peroxidase conjugate	Amersham Pharmacia	RPN 1231
Sulphuric acid	BDH	10249
TEMED (N',N',N",N" tetramethylethylenediamine)	Sigma	67H0136
3,3',5,5'-Tetramethylbenzidine tablets (TMB)	Sigma	T3405
Tris, (hydroxymethyl) aminomethane, $NH_2C(CH_2OH)_3$	Sigma	T6066

Tris ([hydroxymethyl]aminomethane) Borate EDTA buffer (10X)	Sigma	T4415
TRIZMA base	Sigma	T8524
Tween – 20 (polyoxyethylene (20) sorbitan monolaurate)	Sigma	P1379
Universal heteroduplex generator (UHG)	Gift of J.Bidwell and N.Wood, Bristol	

2.1.2 Equipment

Bio-Rad Mini-Protean II gel electrophoresis system with power pack (200V).	Bio-Rad, Hercules, California, USA
Agarose gel electrophoresis apparatus.	Northumbria Biologicals
Hybaid PCR machine	Hybaid, Oxford, UK
MJ Tetrad DNA engine Thermocycler	Applied Biosystems, UK
Thermohybaid Omniultra polycarbonated microplates	Hybaid, Oxford, UK

2.1.3 Buffer solutions

General buffers	**Phosphate buffered saline (PBS), pH 7.3** NaCl 140.0 mM KCl 2.7 mM Na_2HPO_4 8.0 mM KH_2PO_4 1.5 mM Prepared by the addition of one PBS tablet to 100 ml MilliQ water
	PBS Tween Prepared as above with the addition of 0.5 % (v/v) Tween-20

Protein Electrophoresis Buffers	**10 x Transfer Buffer**	
	Tris	48.0 mM
	Glycine	39.0 mM
	3.75 ml 10 % (w/v) SDS added to 1l of 1 x transfer buffer before use	
	SDS-PAGE running buffer	
	Glycine	190.0 mM
	Tris	25.0 mM
	SDS	0.1 % w/v

	2 x Sample buffer	
	Tris	120.0 mM
	Glycerol	20.0 % (v/v)
	SDS	4.0 % (w/v)
	10 x TBE buffer (Tris-Borate-EDTA buffer)	
	Tris, 0.9M	0.9M
	Boric acid	0.9M
	EDTA	20mM

ELISA Buffers	**ELISA coating buffer, pH 9.6**	
	$NaHCO_3$	35.0 mM
	Na_2CO_3	15.0 mM
	Citrate / phosphate buffer, pH 5.0	
	Citric acid ($C_6H_8O_7.H_2O$)	0.1 M
	Na_2HPO_4	0.2 M
	TMB ELISA substrate buffer	
	Citrate / phosphate buffer	20.0 ml
	TMB tablets	4

	H_2O_2	0.015 % (v/v)
Whole blood culture	**Wash buffer**	
	PBS	500mls
	BSA	0.5%
	Sodium azide	0.02%

FITC conjugation of antibody	**'A' mix**		
	Na2CO3 (anhydrous)	5.8 ml	5.3% (w/v)
	NaHCO3	10.0 ml	4.2% (w/v)
	Conjugation buffer		
	'A' mix	1 part	
	0.1 M NaCl	9 parts	
	pH of final mixture 9.5		

2.1.4 Antibodies

Name	Company
Anti-human MBL, mouse monoclonal IgG clone 131-1	State serum Institute, Copenhagen, Denmark A gift from Dr Claus Koch
Mouse IgG1 negative control	Serotec Ltd, Oxford
FITC conjugated anti-human CD14 (mouse IgG2a)	Serotec Ltd, Oxford
FITC conjugated isotype control (mouse IgG2a)	Serotec Ltd, Oxford
Phycoerythrin (PE) conjugated anti human (mouse IgG1) TNF-α IL-6 IL-1ß	 Becton Dickinson, Oxford Becton Dickinson, Oxford R & D systems
PE conjugated isotype control	Becton Dickinson, Oxford

2.1.5 Cytokine Assays

Name	Company
TNF-α ELISA kit	Bioscience, Cambridge
IL-6 ELISA kit	Bioscience, Cambridge
IL-1ß ELISA kit	Bioscience, Cambridge

2.2 Clinical Methods

2.2.1 Study design & recruitment

The clinical studies described in this thesis were designed and commenced at the beginning of my research and were continued, where appropriate, for the subsequent years. I was responsible for patient recruitment at GOSH and Dr J Davies for patients at the Royal Brompton Hospital. This involved identifying suitable patients, approaching parents and children, discussion of the study, asking for consent for participation in the study, allowing time for consideration and returning to obtain written consent. I then transferred samples to the laboratory and processed them as described in the relevant sections.

2.2.2 Ethical applications and approval

All ethical applications, consent and assent forms, parent and child information leaflets for all of the clinical studies at GOSH described here were done in their entirety by myself. For the parts of the work undertaken at The Royal Brompton Hospital the principle investigator was Dr Jane Davies and ethics applications were done by her. All studies were approved by the Ethics Committees prior to their commencement. All parents or carers gave informed consent for participation.

2.2.3 Patient data collection

In order to interpret results, detailed and accurate phenotypic data on each patient were required. The patient data presented in the following chapters combines clinical and laboratory diagnostic results, patient demographic data including age, sex and race, microbiological data and for the study of children in PICU (chapter 6), daily review of the Intensive Care bedside electronic charting system (Care Vue, Hewlett Packard, USA). Patient data were anonymized and stored in a password protected excel

spreadsheet according to the data protection policy of University College London. In addition, for the PICU study where a large amount of data were accrued, a purpose designed Access Data Base (Microsoft Office'98/XP) was developed for the sole use of the small PICU research team which was again password protected.

2.2.4 Blood collection and processing

2-3 mls of whole blood was collected from subjects via an in-situ arterial or venous line or by venupuncture, at a time when a clinical sample was being taken to prevent additional line sampling or venupuncture procedures. Blood was divided into an ethylenediaminetetraacetic acid (EDTA) and a serum collection tube. Whole blood in EDTA was stored at -20°C until DNA extraction was performed. Serum samples were spun, separated and the serum stored in aliquots at -80°C until analyzed.

2.2.5 Diagnosis of CF

In both GOSH and the Brompton the diagnosis of CF was usually made by a positive sweat test and confirmatory genotyping. In some cases babies had been initially identified by a high IRT on newborn screening, or after meconium ileus. Borderline cases had extended genotyping and/or nasal potential difference performed.

2.2.6 Statistical Analysis

The statistical analysis of data are described separately in the methods section of each chapter.

2.3 Laboratory Methods

2.3.1 MBL Enzyme Linked Immunosorbant Assay (ELISA)

The principle of this ELISA is as follows: microwell plates are coated with a monoclonal antibody against the carbohydrate recognition domain of MBL. Bound MBL is detected with the same antibody that has been labelled with biotin, followed by the addition of horseradish peroxidase (HRP) conjugated to streptavidin and subsequent incubation with a colour changing substrate.

2.3.1.1 MBL ELISA used for clinical cohort studies

For all clinical cohorts MBL levels in serum and bronchoalveolar lavage fluid were determined by a symmetrical sandwich ELISA using a kit from the State Serum Institute, Copenhagen, Denmark according to the manufacturer's instructions (Fig 2.1). This kit used the same capture and detection antibody, both binding to the carbohydrate recognition domain (CRD) of MBL. Patient serum was diluted 1 in 10 to 1 in 200 depending on the expected value (if known by genotyping). BAL samples were used neat or diluted 1 in 2. The standard curve was performed with provided calibrators of 0, 0.5, 1, 2, 5, 10, 20 and 40 ng/mL. This kit was chosen as it was being used in many other clinical cohort studies worldwide allowing exact comparison of levels between different research groups. *Intraplate* variation using the kit was < 4% and *interplate* variability on the same or different days was <7%

2.3.1.2 "In-house" MBL ELISA

To determine serial levels of MBL in individual patients over time, an "in-house" ELISA method was used. This was done as it was more cost effective and provided experience of the modification of a previously designed ELISA system. The method used was that of a symmetrical sandwich ELISA previously described (Lipscombe et

al., 1996) and is depicted in Fig 2.1. At each incubation step a volume of 100µl was used, all reagents were diluted in PBS and each washing step consisted of three washes with PBS-Tween (containing PBS and 0.05% Tween-20 (PBS-T)) using an ELISA plate washer unless otherwise stated. Immulon-2 plates were coated with PBS containing 1 µg/ml anti-MBL (clone 131-1), left for 15-17 hours at +4°C and then washed. Serum samples were generally diluted 1/20 or1/200 in PBS and added to appropriate wells. To subsequently produce a standard curve from which to calculate MBL levels, standards of known values, in doubling dilution were added. These were either those from the SSI kits or nine standards from pooled human serum with a known MBL level of 1600ng/ml, diluted down to 320 ng/ml. The standards in this case were therefore 320,160, 80, 40, 20, 10, 5, 2.5, 1.25 ng/ml respectively. Additional internal standards of known high, intermediate and low MBL sera were also added. All test and standard samples were performed in duplicate. At least one well had no sample added to ensure that no non specific background binding was occurring. Samples were incubated at 37°C for one hour, washed and then bound MBL was detected using a biotinylated anti-MBL added at 1 µg/ml and incubated for one hour at 37°C. The plates were rewashed and 1/1000 dilution of streptavidin horseradish peroxidase (S-HRP) was added prior to re-incubation for one hour at 37°C. After washing, the ELISA was developed using 100 µl of TMB substrate solution. Adding 100 µl 4N H_2SO_4 stopped the reaction and the A_{450} of each well was read using a Dynatech MRX plate reader (Acterna, Aldermaston, UK) with the standards plotted as a sigmoid curve (Revelation software).

Prior to using patient samples this system was optimised using various concentrations of capture anti-MBL, biotinylated anti-MBL and S-HRP (1/500-1/1000 in different combinations) with the above method giving the most reliable and reproducible results.

2.3.1.3 ELISA validation

For each ELISA method a calibration curve was made by plotting the mean of the duplicate optical densities (OD) values for each MBL standard on the y axis with the corresponding MBL concentrations in ng/ml on the x axis. The MBL concentration of each diluted sample was then determined from its mean OD. The concentration of MBL in the undiluted specimen was then calculated by multiplying the result by the dilution factor. On each occasion performed, the results were only taken to be valid if the following were obtained: (i) an adequate standard curve, (ii) adequate quality control and interplate variation, (iii) readings falling within the middle straight part of the sigmoid curve and (iv) coefficient of variation (standard deviation of the replicate responses x 100 / mean of the response) between duplicate samples of <10%. If these criteria were not satisfied, plates or individual readings respectively, were rejected and re run. Acceptable interplate variation was confirmed if the high, medium and low "internal" quality control samples were within 10% of their expected value.

In addition during optimisation, *intraplate* variation of known control samples was determined to ensure that coating of plates was even and the ELISA plate reader was reading all parts of the plate consistently. Again values differing less than 10% of their expected value were accepted. During optimisation a number of problems were encountered and rectified. These included variation of laboratory temperature due to air-conditioning failure resulting in the overdeveloping of plates (and higher than expected MBL values) and the partial failure of the ELISA plate washer resulting in differential washing and high *intraplate* variation of control samples.

Both intraplate and interplate variation were slightly higher with the "in-house" method compared to the kit ELISA and therefore the "in-house" method was ultimately only used for serial measurements on the same patient, ensuring all samples were on the same ELISA plate.

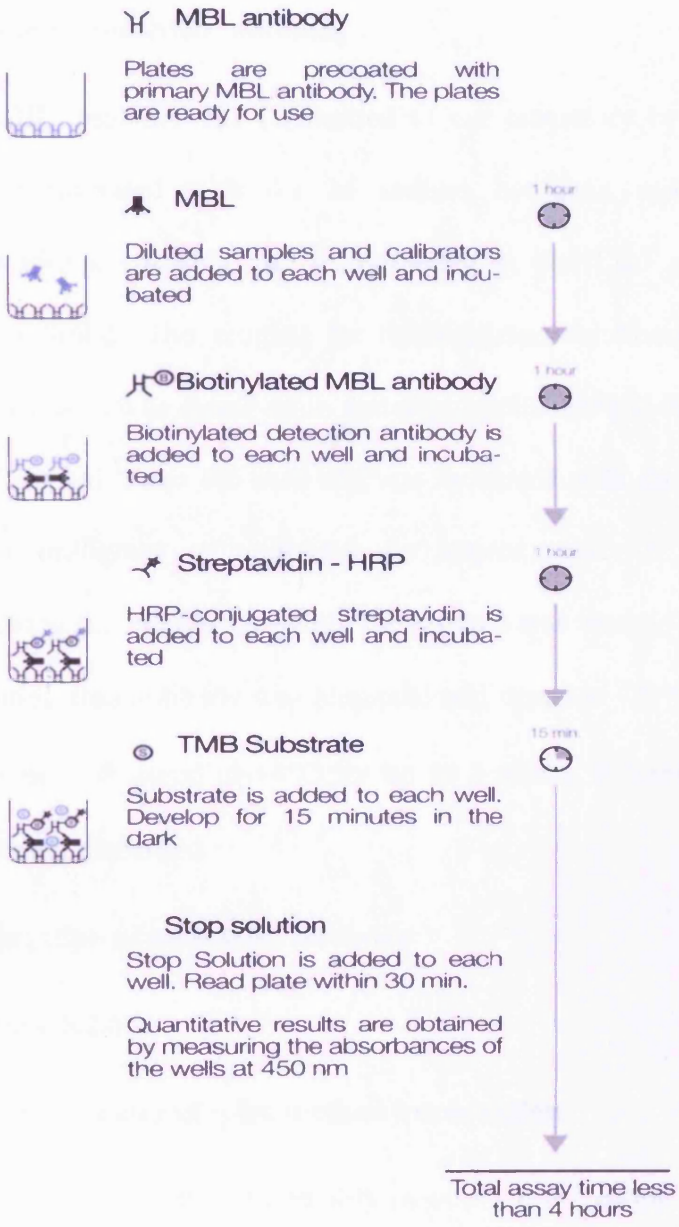


Fig 2.1 Schematic overview of SSI MBL ELISA All plates were actually read within 5 minutes. (Reproduced from SSI information sheet).

2.3.2 Antibody conjugation procedures

2.3.2.1 Biotinylation of anti-MBL antibody

Aliquots of anti-MBL antibody 131.1 (donated to our laboratory by the SSI) to be conjugated were equilibrated with 0.1 M sodium hydrogen carbonate prior to biotinylation by washing, spinning and resuspending in NaHCO3 in Ultrafree MC filters (Millipore, 0.5mls). The reagent for biotinylation, biotinaminocaproate N-hydroxysuccinamide ester, was dissolved in dimethyl sulphoxide (DMSO) to produce a concentration of 1 mg ml^{-1}, and the antibody was incubated with this solution (75 µl ester solution per milligram of antibody) for approximately 5 hours at room temperature. Following the incubation period the product was washed three times with PBS. The final biotinylated antibody was aliquoted and stored at -70°C. Aliquots were defrosted prior to use and stored at +4°C for up to 2 weeks, to prevent freeze-thaw disruption, before being discarded.

2.3.2.2 FITC conjugation of anti-MBL antibody

See chapter 5, section 5.2.6.

2.3.3 MBL Genotyping: heteroduplex method introduction

The heteroduplex method uses PCR to amplify most of exon 1 of the MBL gene. In a separate reaction a synthetic DNA based on exon 1, but containing known deletions, insertions and/or substitutions near the sites of the three mutations is also amplified using the same primers. This synthetic DNA is called Universal Heteroduplex Generator (UHG) (kindly donated by Dr. N. Wood, University of Bristol).

After amplification the two different products are mixed, denatured and allowed to anneal slowly. This allows heteroduplexes (between the two different DNA's) as well as homoduplexes to form, of which the former are retarded on a polyacrylamide gel with respect to homoduplexes of the same length. As two different heteroduplexes are formed for each allele, with differing electrophoretic mobility's, two bands are seen for each allele. Figure 2.2 below is a schematic diagram of the banding pattern seen for each of the ten possible genotypes.

Genotyping was performed separately for the exon 1 mutations and the X/Y promoter polymorphisms using procedures similar to those previously described (Jack *et al.*, 1997; Turner *et al.*, 2000).

2.3.3.1 Preparation of DNA samples for genotyping

Samples for genotyping were stored as whole blood in EDTA at -20°C. DNA was subsequently extracted from 200 µl of thawed whole blood using the Qiagen Blood Amp kit according to the manufacturer's instructions and the final product resuspended in 200 µl of sterile water before being stored at -20°C.

2.3.3.2 PCR amplification of DNA for MBL genotyping

The technique of PCR relies on the fact that DNA can be denatured into single strands by heat, and will anneal with primers and nucleotide bases to reform a double strand on cooling. Primers complimentary to sections of DNA on either side of the sequence to be studied bind to the genomic DNA, and a DNA polymerase adds nucleotides base by base between the 2 primers. The polymerase used in this PCR is derived from the bacterium *Thermus aquaticus (Taq)* and is heat stable, thus replenishment of polymerase after each cycle of heating and cooling was not required. PCR enables very

small amounts of DNA to be increased in quantity until there is sufficient DNA for genotyping to be performed.

Separate PCR reactions were performed on the genomic DNA samples, one to amplify the region of the gene containing the Exon 1 mutations, and one spanning the region of the X/Y promoter polymorphism. Both of these PCRs were also performed for the synthetic UHG.

Genomic DNA was PCR-amplified for **exon 1** in a 20 µl reaction using 5 µl of aqueous DNA (approximately 10 ng μl^{-1}), 0.6 µl each of 50 µM RMBL (5'-CCAACACGTACCTGGTTCC-3') and LMBL (5'-CTGTGACCTGTGAGGATGC-3') primers (Sigma-Genosys, Pampisford, UK), 2 µl of 10x PCR buffer (provided with AmpliTaq Gold enzyme), 2 µl of dNTP mix (2 mM each of dATP, dCTP, dGTP and dTTP), 1.2 µl of 25 mM $MgCl_2$ (provided with AmpliTaq Gold enzyme), 0.2 µl of AmpliTaq Gold DNA polymerase (5u μl^{-1}) and sterile water to make the total volume up to 20 µl.

PCR reactions for the **promoter polymorphisms** were carried out in a similar fashion to the exon 1 procedure, the only exception being substitution of the exon 1 primers for primers for the X/Y promoter; MBLproL (shorty-left, 19 bases) (5'-CTAAGGAGGGGTTCATCTG-3') and MBLproR (shorty-right 21 bases) (5'-AGGCATAAGCCAGCTGGCAAT-3'), (both Sigma-Genosys, Pampisford, UK).

UHG amplification was carried out in larger volumes of 2000 µl for exon 1 and the X/Y promoter with all reagents in the same proportions to the 20 µl genomic reactions except for the UHG DNA where 27 µl were used.

PCR reactions were carried out at 95°C for 15 min, followed by 35 cycles of 95°C for 45 sec, 56°C for 45 sec and 72°C for 45 sec. These cycles were followed by a final extension step of 72°C for 10 min.

The presence of PCR product was confirmed by running 3 µl of product with 1 µl of bromophenol blue loading dye on a 2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide in 1 x TBE running buffer for 1 hour. Gels were visualised under UV light.

2.3.3.3 Heteroduplexing and genotyping

Following confirmation of amplification of DNA, 10 µl of UHG PCR product and 5 µl of loading dye were added to each genomic DNA sample. Samples were heteroduplexed by heating at 95°C for 10 min and then allowing samples to cool to room temperature for 20 min.

2.3.3.4 Detection of haplotype on polyacrilamide gel

Fifteen microlitres of heteroduplex product were loaded onto 20% polyacrylamide gels and run for 15 hr at 150 V in 1 x TBE buffer at 16°C. Gels were stained in 0.5 µg ml^{-1} ethidium bromide in 1 x TBE buffer for 10 min before being visualised under UV light. Characteristic and specific bands were obtained for each haplotype (Fig 2.2).

All genotypes and haplotypes were initially identified by me. Any equivocal results were repeated. They were then all read by a second independent person who was blinded to my diagnosis. 100 % concurrence was seen between both sets of results.

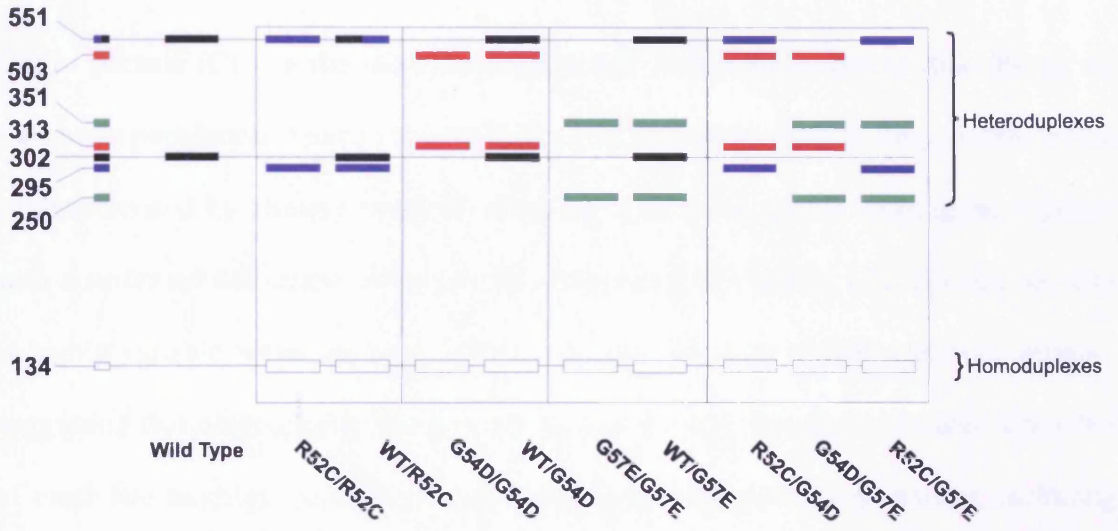


Fig 2.2 Schematic diagram showing the banding patterns for each of the possible MBL exon 1 genotypes. Homozygotes for wild type or a mutation reveal 2 bands, whilst heterozygotes or compound homozygotes demonstrate 4 bands. The apparent size in base pairs of each of the bands is shown on the left. The X/Y promoter polymorphisms also had a characteristic pattern. (This diagram was kindly provided by Dr R. Hamvas).

CHAPTER 3

The Role of Mannose Binding Lectin in Cystic Fibrosis Lung Disease

3.1 Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in the Caucasian population. Most of the morbidity and mortality relates to lung disease which is characterised by chronic bacterial infection, with quite specific pathogens, together with a sustained deleterious inflammatory response (see Chapter 1.7). Disease severity is highly variable, even amongst individuals with the same *CFTR* mutation, strongly suggesting that other genetic factors may modify the CF phenotype. To date a number of candidate modifier genes have been associated with pulmonary disease, including *TNF-α* (Hull and Thomson, 1998), *nitric oxide synthase 1* (*NOS1*) (Texereau et al., 2004), *transforming growth factor beta* (*TGF-β*) (Arkwright et al., 2000) and *surfactant protein A1 and A2 genes* (Choi et al., 2006). Unfortunately many association studies have been based on small numbers and consequently multiple studies have given conflicting results (Cutting, 2005).

Prior to the commencement of this work, two compelling studies had just reported that *MBL-2* was a modifier of lung disease in CF. The first of these by Garred *et al* demonstrated, in 149 Danish patients, that lung function was significantly reduced in patients with chronic *Pseudomonas aeruginosa* infection possessing either one or two structural MBL-2 gene mutations (A/O or O/O) compared with normal wild type homozygotes (A/A) (Garred et al., 1999b). Patients with variant alleles also had a significantly increased risk of end stage lung disease (death or transplant) compared

with those who had no mutations. Specifically, the predicted age of survival in those carrying variant alleles was reduced by 8 years compared to wild type individuals. In addition there was some preliminary evidence that patients were also at risk of acquiring the potentially-lethal organism, *Burkholderia cepacia,* with 7/10 of patients infected with *B. cepacia* carrying an MBL variant allelle. *P.aeruginosa* carriage was not affected *per-se* by MBL genotype however there was a trend towards carriers of variant alleles being younger at the onset of chronic carriage. A second study by Gabolde (Gabolde et al., 1999), of 164 French CF patients homozygous for the ΔF508 mutation, matched 11 MBL-deficient adults (O/O) with homozygous sufficient controls (A/A). They found a reduction in lung function only in adults with two *MBL-2* mutations. In addition a trend for more O/O than A/A patients to be colonised with *P.aeruginosa* was demonstrated. The same group subsequently also found that CF liver cirrhosis is significantly associated with the presence of MBL mutations (homozygous or compound heterozygote) (Gabolde et al., 2001).

Whether the effect of MBL on CF lung disease is seen just in those homozygous (or compound heterozygous) for a mutation or in the larger number of MBL heterozygote patients as well, would have important implications if MBL replacement therapy were to be developed as a treatment for CF. In addition both of these clinical studies focused on older children and adults with median ages of 16.2 and 19 years respectively and it was unclear whether MBL also acts as a modifier gene in children.

The aim of this part of my work was to investigate whether MBL plays a role in CF lung disease in a different geographical population (UK) to those previously described and to determine if the effect is also seen in children. Two separate hospitals were chosen so that the findings in two independent cohorts could be compared. This chapter describes the two clinical studies addressing these questions.

3.2 Methods

3.2.1 Patient Population GOSH & RBH

Children were recruited from the paediatric CF clinic at Great Ormond Street Hospital (GOSH) between December 2001 and December 2002 and adults and children from respective clinics at the Royal Brompton Hospital (RBH) during 2000 and 2002. After informed consent, at the time of a routine blood test, an extra 1-3 ml of blood was taken from all patients from whom sufficient could be obtained. This was mainly done at annual review when patients were as well as possible apart from 4 children at GOSH, 3 of whom were admitted for routine elective intravenous antibiotics and one who was admitted unwell. Retrospective clinical data were obtained from the CF database, patients' hospital notes, the lung function laboratory and computerised microbiology reports. Microbiological data recorded included the age at first isolation of *P. aeruginosa* and *B. cepacia* complex. *S. aureus* was not included due to the frequent use of prophylactic anti-staphylococcal medication. *CFTR* genotype was recorded if this was available (performed in the DNA Laboratory, Kennedy Galton Centre, Harrow, UK). In addition, prospective follow up information on all children from GOSH was obtained by myself, from study entry until December 2005. If they had transferred to adult clinics or moved to other areas of England I contacted the relevant unit at the end of the study in December 2005 to obtain information regarding death/ transplantation and/or acquisition of *B. cepacia* infection.

All clinical information obtained was organised in an excel spreadsheet and subsequently transferred into the statistical programmes SPSS (Version 12.00) and/or PRISM (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA) prior to analysis.

3.2.2 Serial MBL serum measurements

A small number of children from GOSH, who were admitted either acutely or for elective intravenous antibiotics, were recruited for serial measurements of MBL. Blood samples were obtained whenever a routine clinical sample was taken to avoid unneccesary venupuncture or access of central lines.

3.2.3 Ethical Approval

The studies were approved by the Ethics Committees of Great Ormond Street and Royal Brompton Hospitals. All parents or carers, and children where old enough, gave informed consent for participation.

3.2.4 Lung Function Testing

Forced expiratory volume in the first second (FEV_1) and forced vital capacity (FVC) were obtained from annual lung function laboratory records. Spirometry was measured using Jaeger MasterScreen and Masterscreen Body systems (Viasys Healthcare, Germany) following ATS spirometry guidelines. The results are expressed as percent of predicted values for age using published reference data (Rosenthal et al., 1993).

For the paediatric cohort at GOSH, FEV_1 and FVC were obtained for each year from age 5-16 where available. For children at the Brompton FEV_1 and FVC were obtained for the following approximate time points: 6-7 years, 8-9 years, 10-11 years, 12-13 years and 14-16 years. For adults, annual values were recorded, where available, from 1990-2002.

3.2.5 Sample processing

Preparation and amplification of genomic DNA, and *MBL-2* haplotyping were performed as previously described (Chapter 2.3.3). MBL serum levels for the whole cohort were measured by a symmetrical sandwich ELISA using commercial kits

(Antibody Shop, Copenhagen, Denmark) according to the manufacturer's instructions. Serial MBL levels on individual patients were performed using the "in house" ELISA (section 2.3.1).

3.2.6 Statistical analysis

Data were analysed using SPSS v 12.00 and/or PRISM (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA). Differences in serum MBL levels between MBL groups were analysed with the Kruskal Wallis (KW) test. Lung fuction test (LFT) data were normally distributed and therefore analysed by one-way ANOVA and presented as mean +/- 1 SEM. Observed and expected genotype frequencies were analysed by the Chi square ($\chi 2$) test.

3.3 Results

Patient demographics and *MBL-2* haplotype results are shown for the GOSH & Brompton cohort separately to permit comparison between the 2 cohorts. Data are also presented separately for children and adults. Prolonged prospective follow up at GOSH enabled survival/transplantation data to be analysed for this group only.

3.3.1 Patient demographics

Great Ormond Street Hospital cohort

122 consecutive children and parents were approached of whom one refused consent and one had insufficient blood available for a research sample to be taken. This resulted in 120 children with a presumed diagnosis of CF being available to study. A diagnosis of CF was made on a clinical history and examination consistent with CF and a positive sweat test +/- a known CF genotype or a neonatal screening test with confirmatory genotyping or sweat test. During the period of follow up it became clear that two of the children did not infact have CF. Both had presented with respiratory infections and borderline sweat tests. Neither had a CF mutation detected and both were subsequently found to have normal nasal potential differences (Delmarco et al., 1997;Wilschanski et al., 2001). On subsequent investigation both had hypogammaglobulinaemia and interestingly both were MBL genotype OO, making a diagnosis of minor immunodeficiency likely as a cause for their symptoms. These 2 were therefore removed from all analyses. Of the remaining 118 cases, 55 (47%) were male and the mean age at study entry was 9± 0.4 years. The study group included 3 sibling pairs. *CFTR* genotype data were available on 114 (96.6%) children. 79 (69.3%) were homozygous for the ΔF508 mutation, 29 (25.4%) were compound heterozygotes with one copy of ΔF508 and the remaining 6 (5.3%) possessed 2 other mutations

(G542X/G542X, G551D/R1158X, G551D/R5560T, Y569D/Y569D, 1461in4/-, 6551D/-). Lung function data were analysed on all patients and also for the ΔF508 homozygotes only.

Royal Brompton hospital cohort

Blood samples were available from 298 (57% male) adults aged 29.7±0.5 years. *CFTR* genotype data were available on 269 (90.3%) of these as follows: Δ/Δ 48%, Δ/other 44.6%, other/other 7.4%. A total of 260 children, (48%) male, with a mean age of 8.5±0.3 years were included. In 228 (88%) children *CFTR* data were available as follows: Δ/Δ 58.8%, Δ/other 34.6% and other/other 6.6%.

3.3.2 Mannose binding lectin genotypes

Great Ormond Street Hospital cohort

Genotype results were available for 113 of the 118 patients. No EDTA blood was available for genotyping in 3 individuals and 2 samples failed genotype testing on 3 attempts, probably due to insufficient amount or poor quality of DNA. These 5 patients all had MBL serum results. Of the 118 patients serum was also available for MBL determination in 99 patients In 94 cases both MBL serum levels and *MBL-2* haplotype data were available for correlation. Overall 63/113 (55.7%) had no structural *MBL-2* gene mutation (ie. wild-type and designated conventionally as A/A). 42 (37.2 %) of subjects were heterozygous for structural mutations (designated A/O) and 8 (7.1%) were either homozygous for one mutation or were compound heterozygotes (both designated O/O). This genotype distribution was similar to that observed in the Brompton CF cohort (below) and another UK study of healthy subjects (Mead et al., 1997) (p=1.0 ANOVA), Table 3.1A. Allele frequencies were as follows; A 0.75, B 0.174, C 0.036, D

0.040 which were not statistically different (p=0.9 $\chi 2$) from those seen in the study by Meads et al, Table 3.1B.

Royal Brompton hospital cohort

Both MBL serum levels and *MBL-2* haplotype data were available in 522 cases; in 7 cases only a genotype was obtained and in 25 only protein levels were available. The frequencies of mutations in both exon 1 and the promoter region did not differ between adults and children, and were similar to those observed in other studies of both healthy subjects and patients with CF (Garred et al., 1999b). Overall, 61.1% had no structural mutations, 35.2% were heterozygous, and 3.8% were either homozygous or compound heterozygous (Table 3.1 A). In patients with structural gene mutations, the B allele was found on 71% of chromosomes, with the other mutations occurring less frequently (C 11%, D 18%). The low-expressing X promoter polymorphism was found in association with 30.6% of wild-type structural alleles.

Genotype	Frequency in GOSH CF population (%)	Frequency in Brompton CF population (%)	Frequency in UK "control" population (%)*
A/A	55.7	61.1	59.6
A/O	37.2	35.2	35.8
O/O	7.1	3.8	4.6
A/O + O/O	44.3	39	40.4

Table 3.1 A

Allele	Frequency in GOSH CF population	Frequency in UK "control" population *
Wild type (A)	0.743	0.775
Codon 52 mutation (D)	0.040	0.066
Codon 54 mutation (B)	0.181	0.144
Codon 57 mutation (C)	0.035	0.015

Table 3.1 B

Table 3.1 The frequencies of (A) MBL genotypes in the GOSH and Brompton cohorts and (B) MBL alleles in the GOSH cohort, compared to those in a UK "control" population. These frequencies are not significantly different (p=1.0, ANOVA for genotype and p=0.9, $\chi 2$ for allele frequencies) between any of the groups studied. *(Mead et al., 1997).

3.3.3 Relationship of MBL haplotype to serum MBL level

There was a significant correlation between serum MBL level and haplotypes in all cohorts ($p<0.001$ Kruskal Wallis). The highest protein levels were seen in patients with wild type structural alleles, and the lowest in individuals homozygous or compound heterozygous for structural mutations, and in heterozygotes with the X promoter mutation. Data was similar for all cohorts and only the GOSH data are presented in Fig 3.1.

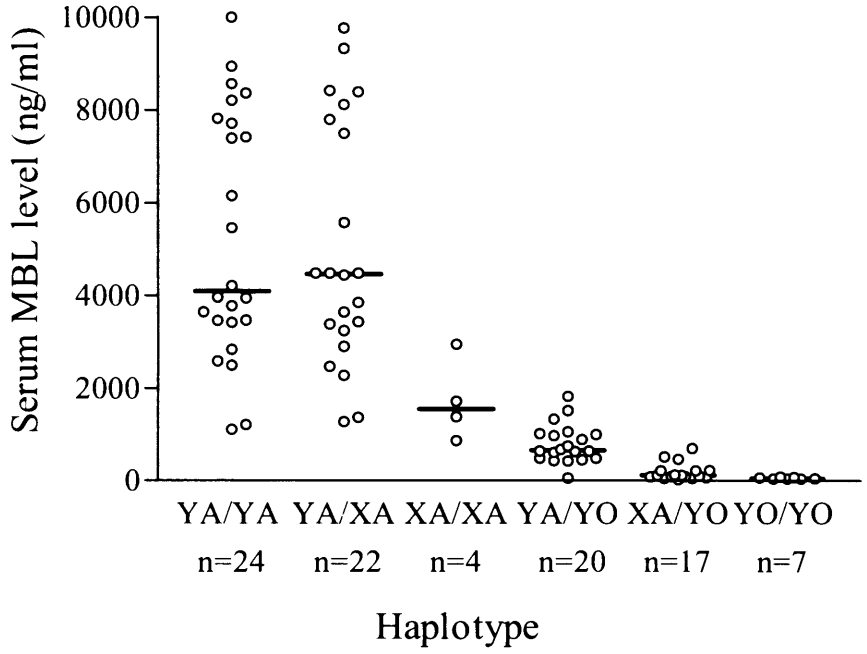


Fig 3.1 Relationship between MBL serum level and MBL haplotypes for GOSH patients. There was a significant correlation between serum MBL level and MBL haplotype ($p<0.001$ Kruskal Wallis) with wild type genotypes (A/A) having higher levels than heterozygotes (A/O) and homozygotes (O/O). The promoter polymorphism X/Y also significantly influenced the serum MBL level as shown.

3.3.4 Death and transplantation in GOSH cohort

During the period of follow up of the GOSH paediatric cohort (4 years prospectively from Dec 2001-Dec 2005), 3 children died, 2 had heart-lung transplants and 1 required a liver transplant. MBL variant alleles were significantly overrepresented in these patients, (5/6 compared to 42/107 non-transplants/survivors) p=0.03, $\chi 2$ test. All three who died were MBL insufficient with genotypes and serum levels as follows: YO/YO, 44ng/ml; XA/YO, 74 ng/ml and YA/YO, serum unavailable. One of the two patients undergoing heart-lung transplantation was MBL insufficient; XA/YO, 57ng/ml, the other sufficient XAXA, 2953 ng/ml and the child requiring liver transplantation was also MBL deficient; XA/YO: 94 ng/ml. All deaths and lung transplants were for respiratory complications related to CF and the child with the liver transplant had CF related liver disease. These data, together with age at death, are shown in Table 3.2. Three of these patients who were over the age of 16, died or received a transplant after transfer to an adult unit. The children who died or had a transplant were, unsurprisingly, some of the oldest children in the cohort. 42 children were over the age of 14 years with both LFT/outcome data and a known MBL genotype. Of these older children all 4 who died or had a heart/lung transplant possessed an MBL variant allele compared to 14/38 of those who survived. The effect of MBL genotype on risk of death/transplant therefore was even more statistically significant when comparing just the older children in the cohort (p=0.015, $\chi 2$ test).

Patient No	MBL genotype	MBL serum level(ng/ml)	Death or transplantation	Age at death or transplant (years)
1	O/O	44	Died	14.8
2	XA/O	74	Died	16.1
3	YA/O	n/a	Died	16.8
4	XA/O	57	HLT	17.1
5	XA/XA	2953	HLT	12.1
6	XA/O	94	Liver transplant	14.2

Table 3.2 Relationship of MBL genotype to death or transplantation in the GOSH paediatric cohort. MBL variant alleles are significantly overrepresented in the patients who died or received a transplant, $p=0.03$, $\chi 2$ test.

HLT= heart lung transplant, n/a= not available

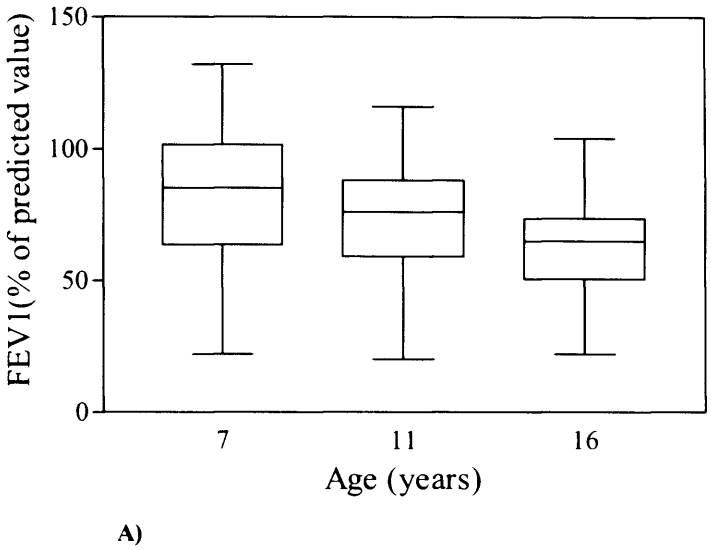
3.3.5 Lung function tests and MBL genotype

Great Ormond Street Hospital paediatric cohort

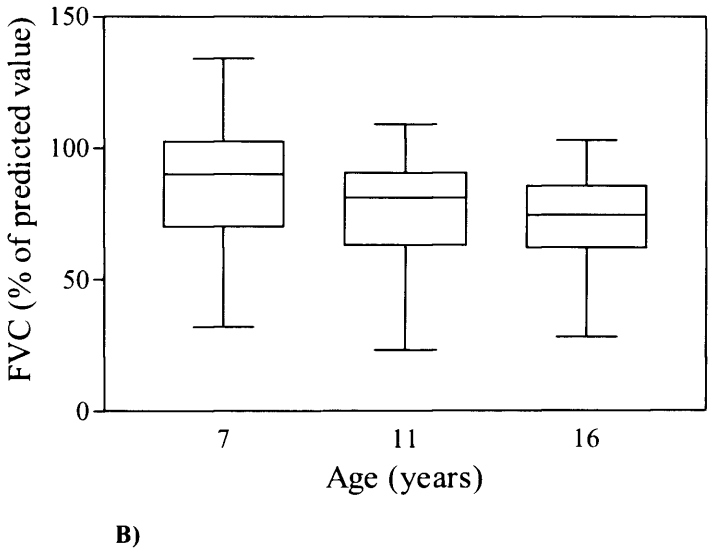
111/118 children had lung function measured on at least one occasion with the other 7 children being too young to perform the test. As expected in children with CF both FEV_1 and FVC were lower in older children than younger ones (p=0.0004 and 0.0012 respectively, Kruskal Wallis test) (Fig 3.2). Lung function was therefore analysed according to MBL groups for early and late childhood. No gender difference was seen in FEV_1 or FVC at any age analysed (Fig 3.3), and therefore gender was not taken into account in further analysis. As MBL haplotype groups were small FEV_1 and FVC were analysed according to MBL genotypes A/A vs A/O vs O/O for the following age groups: 7 years, 11 years and the mean FEV_1 and FVC for ages 5-11 years. Only 4 of the OO children were over the age of 12 and therefore FEV_1 and FVC for the 16 year olds and mean FEV_1 and FVC for 12-16 years was analysed for MBL genotype A/A vs A/O *and* O/O combined. In addition the effect of the X-promotor polymorphism and ΔF508 homozygosity was also determined for each of the above age groups.

No significant difference in lung function was seen between the different MBL groups, at any age in childhood (full data not shown) although it was noted at age 5-11 years that those with genotype A/O and O/O had slightly higher FEV1 and FVCs than those in the A/A group, and by age 12-16 this was reversed, (Fig 3.4 and 3.5). No effect of the X-promotor polymorphism was seen and limiting analysis to only those with CF genotype ΔF508 / ΔF508 did not alter any of these findings (therefore data not presented). Three sibling pairs were present in this cohort, with two pairs possessing different MBL genotypes (both pairs YA/O and O/O, although different MBL mutations). In both pairs lung function was worse in the O/O patient than the YA/O

patient at the same age (pair 1, each year from age 4-11, pair 2, aged 5 only). However, factors such as birth order, age at diagnosis and any differences in treatment of these siblings are likely to be as influential as MBL genotype in difference in subsequent lung function.



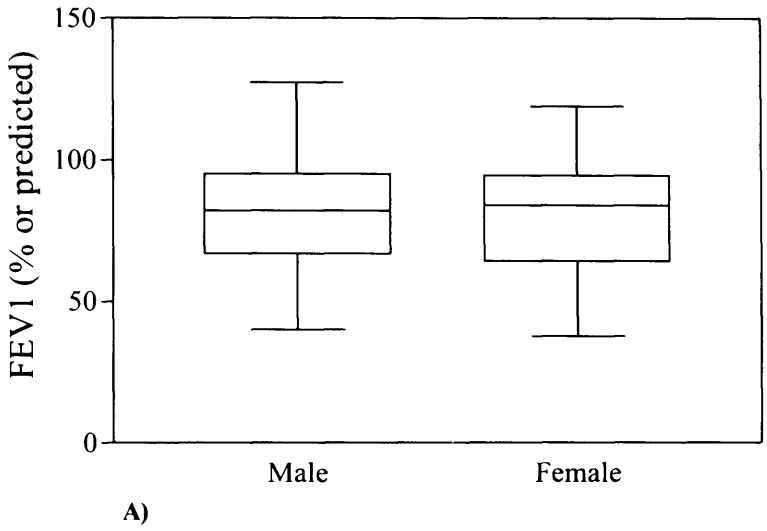
A)



B)

Fig 3.2 A) FEV_1 and B) FVC according to age in patients with CF.

As expected median FEV_1 and FVC both significantly reduced as age increased; p=0.0004 and 0.0012 respectively by Kruskal Wallis test.



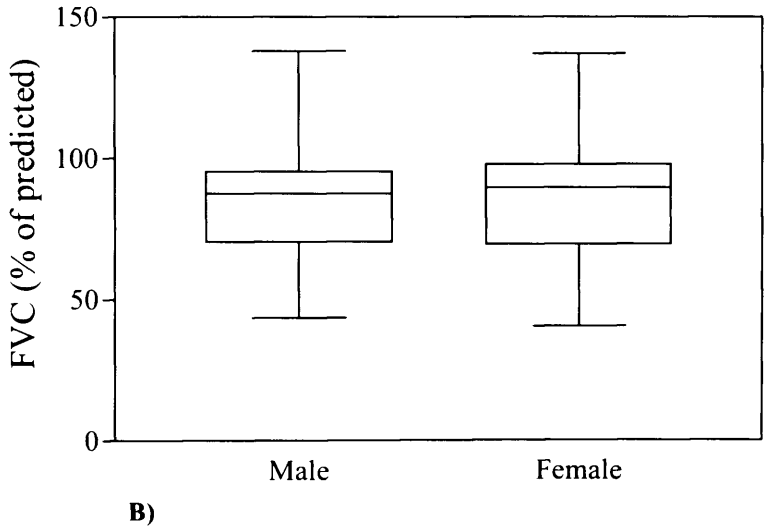


Fig 3.3 A) FEV_1 and B) FVC of all ages 5-16 years, according to gender.

There was no significant difference between median FEV_1 and FVC at ages 5-11, 12-16 (both not shown) or 5-16 years (shown here) according to gender of patients.

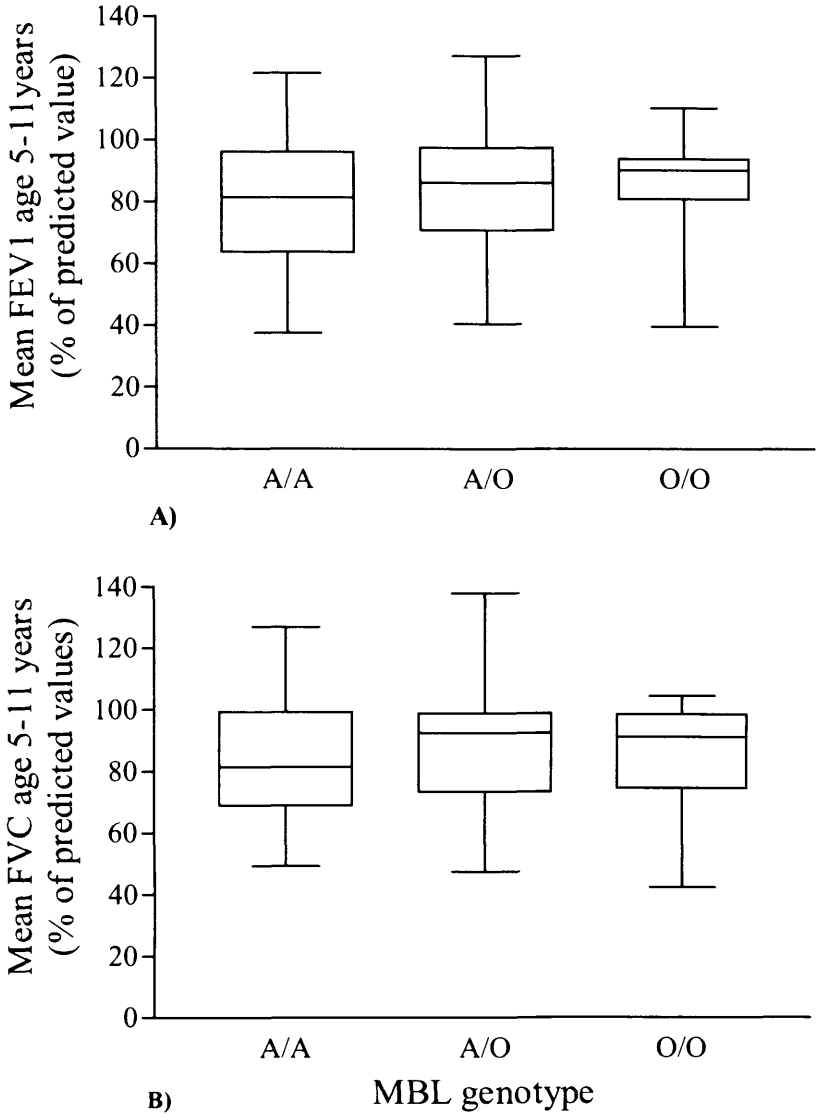


Fig 3.4 The relationship between MBL genotype and the mean A) FEV_1 and B) FVC for ages 5-11 years.

All 8 of the children with MBL genotype O/O had LFT's at this age and therefore this group, although small, was kept separate for analysis. There was no significant difference between MBL wild type individuals and those hetero or homozygous for an MBL variant allele, in relation to FEV_1 and FVC, at this age.

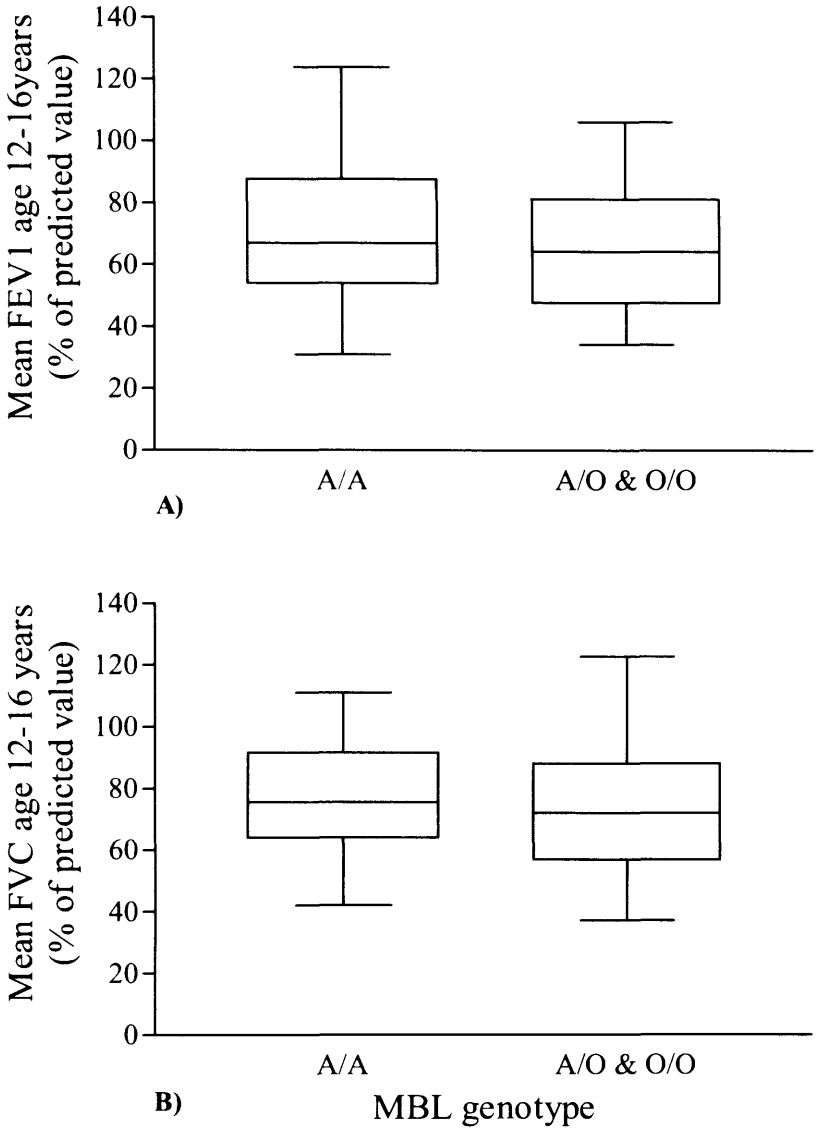


Fig 3.5 The relationship between MBL genotype and the mean A) FEV1 and B) FVC for ages 12-16 years.

As only 4 children with MBL genotype O/O had LFTs above the age of 12 and only one at age 16 years the O/O genotype was combined with the other MBL deficient group A/O for analysis. There was no significant difference between between MBL wild type individuals and those with a MBL variant allele in relation to FEV_1 and FVC at this age.

Royal Brompton hospital paediatric cohort

191 children were old enough to have lung function measured on at least one occasion of whom 186 had an MBL genotype available. As none of the O/O children was over the age of 12 years, all comparisons were made for lung function obtained between the ages of 7 and 11 years (mean age A/A 9.7, A/O 9.7, O/O 8.6 years, ANOVA p=0.3). Results were identical to the GOSH cohort with neither mean FEV_1 or FVC being reduced at this age in children possessing the O/O haplotype (Fig 3.6) and there was no effect of the X promoter polymorphism (data not shown).

Royal Brompton hospital adult cohort

A mean of 6.1 lung function recordings (range 1 to 13) was available on 273 haplotyped adults over a period of 6.5 years. Mean FEV_1 was significantly associated with the *MBL-2* haplotype, the lowest values being observed in individuals possessing two structural mutations (ANOVA $p<0.05$). A similar pattern was seen for mean FVC (Fig 3.7). Neither parameter was reduced significantly in heterozygotes. No effect was seen of the low-expressing X promoter polymorphism on lung function in either the homozygous sufficient or heterozygous groups (data not shown).

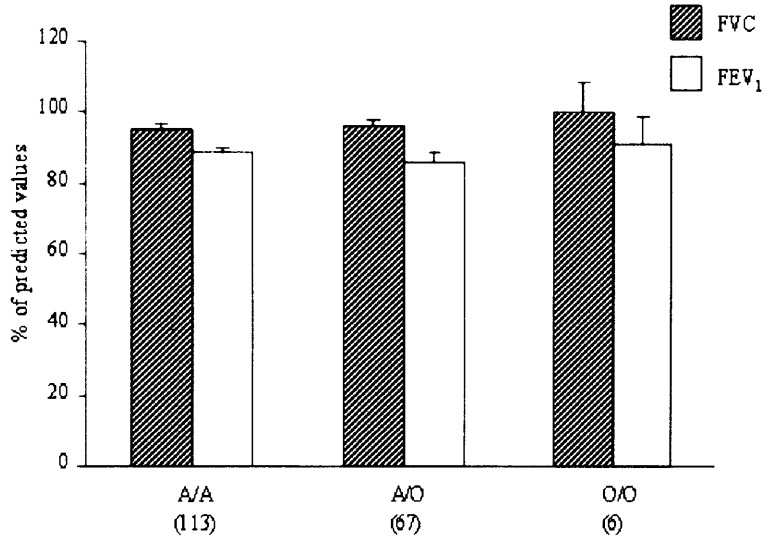


Fig 3.6 The relationship between MBL genotype and FEV_1 and FVC for 186 *children* from the Royal Brompton Hospital. No child was over 12 years so the mean FEV_1 and FVC from age 7-11 years was used. There were no significant differences in lung function measurements between those with different MBL genotypes.

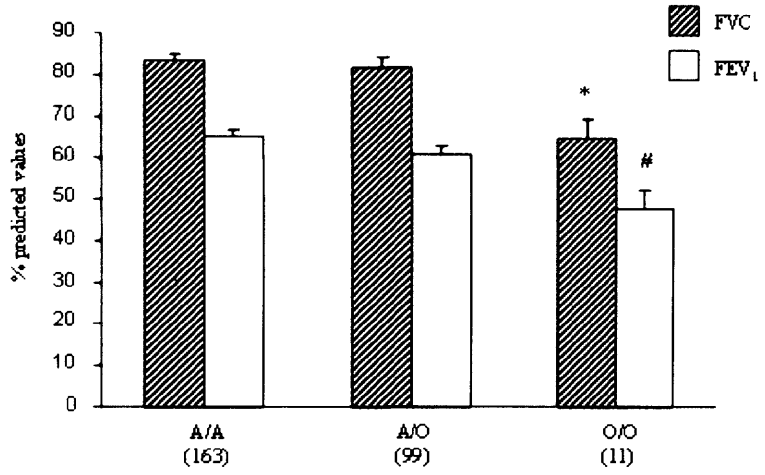


Fig 3.7 The relationship between MBL genotype and FEV_1 and FVC for 273 *adults* from the Royal Brompton Hospital. In adults, both FEV_1 and FVC were significantly different across the 3 *MBL-2* haplotype groups (ANOVA $p<0.05$). The lowest values were seen in subjects with the O/O haplotype (* $p<0.05$ vs both other groups; # $p<0.05$ vs A/A subjects). The values in heterozygotes were not significantly different from those of wild-type patients.

3.3.6 Lung function and Serum MBL levels

Great Ormond Street Hospital paediatric cohort

There was no direct correlation between the MBL serum levels and lung function in the GOSH paediatric cohort (p=0.3, Spearman), as expected from the lack of association with genotype. However when lung function was compared between groups divided on the basis of upper, middle and lower quintiles of MBL serum levels an interesting pattern emerged.

Highest FEV_1 and FVC were observed in the middle quintile group, with patients in both upper and lower quintiles demonstrating lower lung function. This was seen at all ages. Figs 3.8-3.9 show data for ages 5-11 and 16 years respectively. The effect of better lung function in the middle quintile was significant for FVC at age 5-11 years (p= 0.0157 one way ANOVA) with a trend towards significance being seen for FEV_1 age 5-11 (p=0.079). At age 16 this was not significant, maybe inpart due to smaller numbers, FEV_1 age 16 (p=0.19) and FVC age 16 (p=0.26, all using one way ANOVA). Due to different numbers of patients in each MBL quintile, especially small numbers in the older age children, groups were subsequently statistically weighted using the post hoc Turkey test. Following weighting the mean FEV_1 and FVC from ages 5-16 years was also significantly higher in the middle quintile group (both p=<0.0001, one way ANOVA).

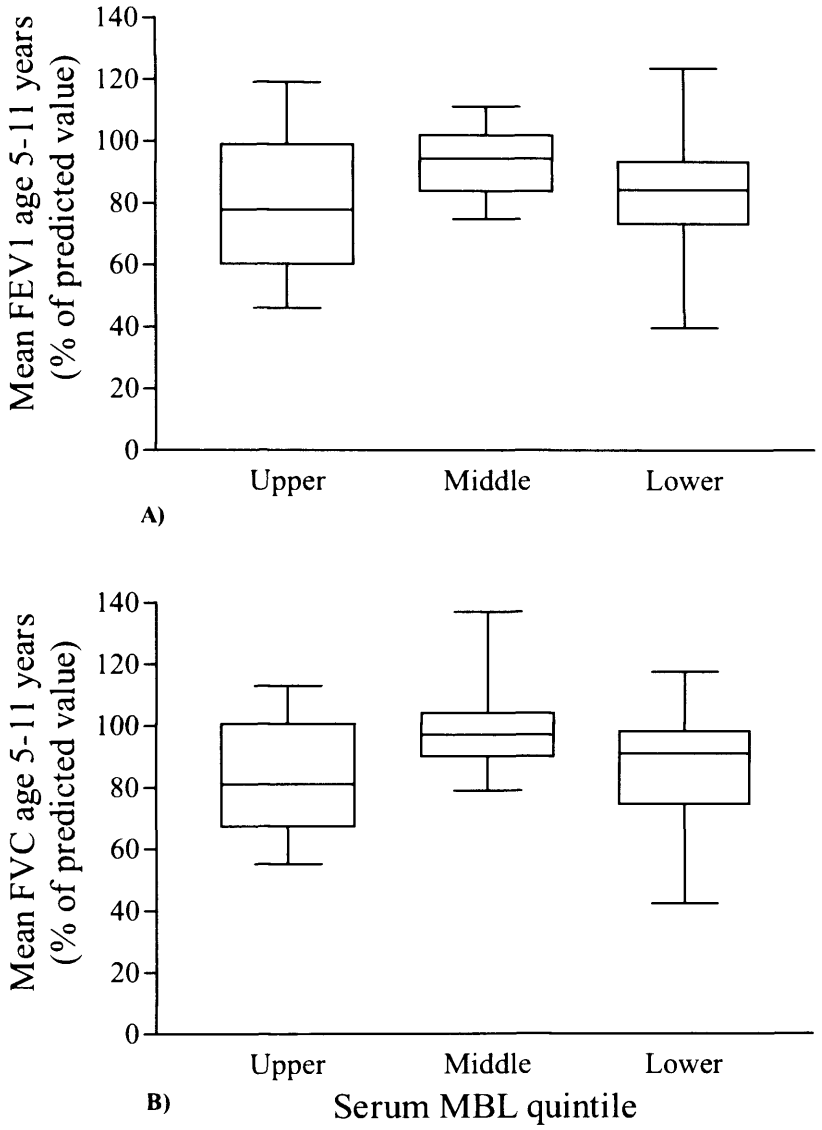


Fig 3.8 The relationship between serum MBL quintile and mean A) FEV_1 and B) FVC for ages 5-11 years.

Patients with MBL levels in the middle quintile had a trend towards a higher FEV1 ($p=0.079$, one way ANOVA) and a significantly higher FVC ($p=0.0157$, one way ANOVA).

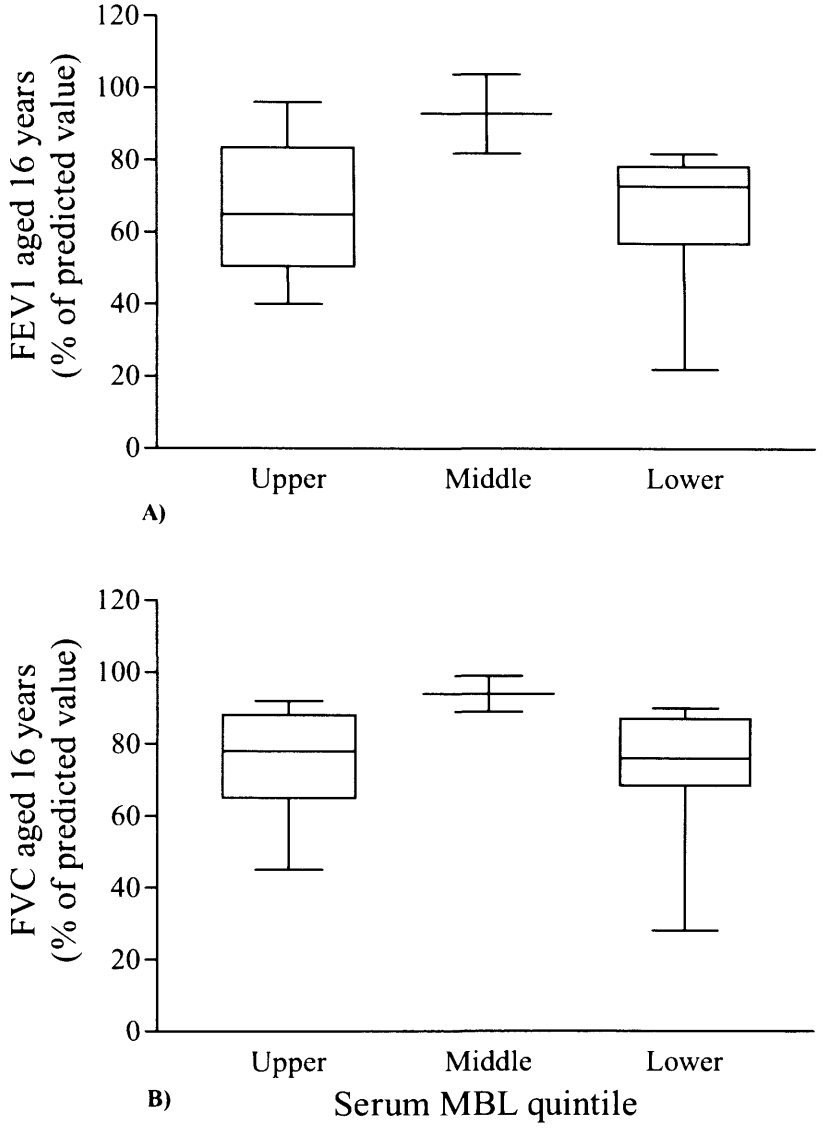


Fig 3.9 The relationship between serum MBL quintile and A) FEV1 and B) FVC at age 16 years.

Patients with MBL levels in the middle quintile had a trend towards a higher FEV1 and FVC ($p=0.19$ and $p=0.26$ respectively, both by the one way ANOVA test).

Royal Brompton hospital paediatric cohort

Lung function was not analysed according to serum MBL levels for this cohort.

Royal Brompton hospital adult cohort

Again despite the close relationship between *MBL-2* haplotype and MBL protein levels in adults, there was no direct correlation between the latter and lung function (p=0.17). To explore this discrepancy, lung function was again compared between groups divided on the basis of MBL serum level into top, middle and bottom quintiles, where an almost identical pattern emerged as in the GOSH paediatric cohort. Highest FEV_1 and FVC were observed in the middle quintile group, with both upper and lower quintiles groups demonstrating lower lung function ($p<0.05$ for FEV1 and p=0.02 for FVC) (Davies et al., 2004).

3.3.7 Infection with *P.aeruginosa* & *B.cepacia* complex

In total 85.8% of GOSH and 85.1% of the Brompton children had experienced at least one positive culture for *P.aeruginosa.* The majority of the adult cohort (86.7%) had had repeated positive cultures for P. aeruginosa. There was no significant difference between *MBL-2* haplotype and age of first *P. aeruginosa* culture (GOSH median age 3.7 (1.5-6.5) years) in any of the cohorts. Due to limited numbers of non-infected individuals further analysis of this effect on the basis of MBL status was not attempted.

Ten children, 3 from GOSH (2.5%) and 7 from the Brompton (2.7%) grew *B.cepacia* at least once each. There was no apparent trend with regard to MBL haplotype (YA/YA x4, YA/XA x3, XAXA, YA/YO and XA/YO). 18/298 (6%) adults were infected with *B.cepacia.*

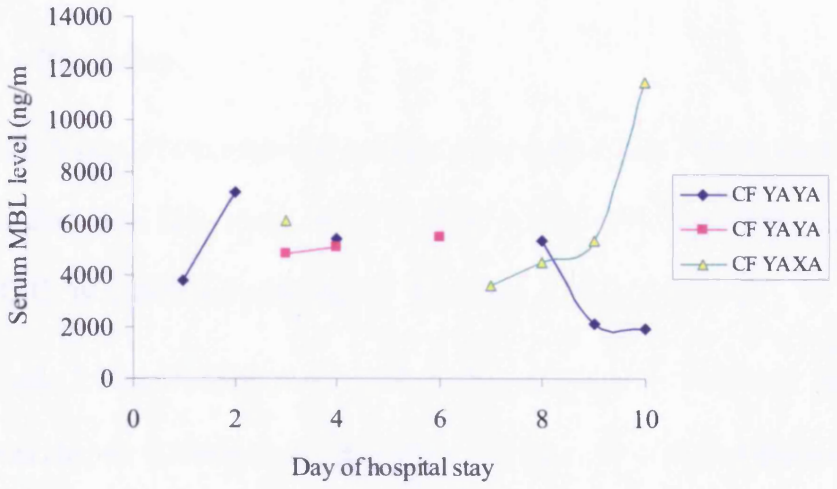
A summary of all data from GOSH patient's is presented in Table 3.3.

MBL genotype	**A/A** n= 63	**A/O & O/O** n=50	**p value**
Age, mean (SEM)	9.23 (0.58)	9.29 (0.63)	n/s
Male sex % (no)	49.2 (31)	48 (24)	n/s
Serum MBL level, median (range) ng/ml	3995 (867-10000)	328 (30-1833)	**<0.0001** MWU
Age at acquisition of pseudomonas median (range) years	3.4 (0-15)	4.15 (0.2-14.5)	n/s
Mean (SEM) of all ages FEV1	78.4 (2.98)	82.2 (3.07)	n/s
Mean (SEM) of all ages FVC	82.5 (2.45)	86.8 (3.09)	n/s
ΔF508 / ΔF508 %(No.)	71.0 (44)	72.3 (34)	n/s
Deaths/transplants % (No.)	1.5 (1)	10 (5)	**0.03** χ^2

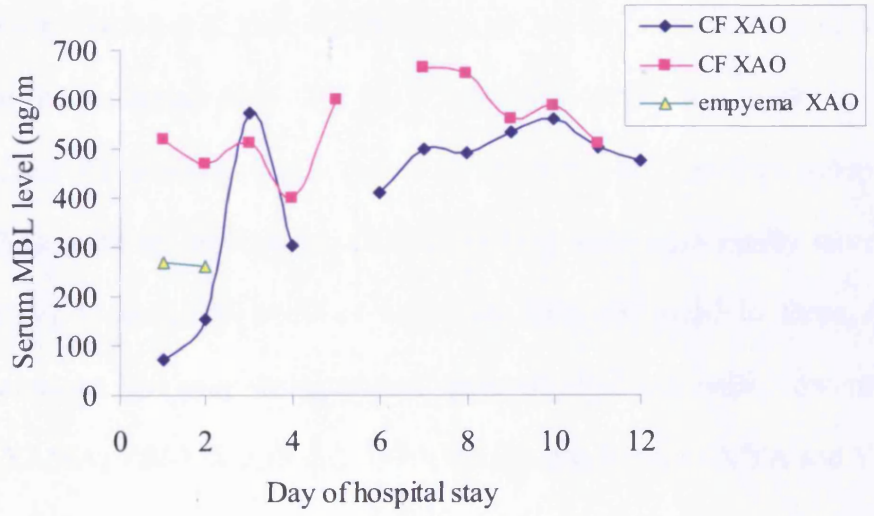
Table 3.3 Table of GOSH patient demographics and all results relating to MBL genotypes A/A vs A/O and O/O. Both MBL serum level and number of deaths or transplants were significantly related to MBL genotype. A significantly higher percentage of CF patients with MBL variant alleles died or were transplanted (p=0.03, $\chi 2$).

3.3.8 Serial MBL measurements

Serial measurements were performed in 5 children with CF and one with empyema (Fig 3.11). Three wild type individuals had marked variation in MBL levels with no obvious pattern (Fig 3.11 A). None of the MBL insufficient patients with genotype XAO (two CF patients and one child with empyema on PICU), mounted an MBL serum level above 700ng/ml (Fig 3.11 B). 1 patient with genotype XAXA, fortuitously had an initial serum level performed at diagnosis during an admission to PICU with meconium ileus. This was 4075 ng/ml. A repeat serum sample one year later when well at annual review, revealed a level of 867 ng/ml, also demonstrating the acute phase response of MBL is usually small, of the order of 3-5 times baseline compared to the large increments seen in other acute phase proteins such as C- reactive protein (CRP) (Thiel et al., 1992).



A) MBL sufficient patients



B) MBL insufficient patients

Fig 3.10 Serial MBL levels for patients during hospital stay. Changes in serum MBL levels are shown during hospital admission for **A)** 3 CF patients with MBL sufficiency and and **B)** 2 CF patients and one child with empyema on PICU, all MBL insufficient with genotype XAO. None of the heterozygote XAO patients can mount an MBL serum level above 700ng/ml. (note scale change in **A)** and **B)**). Data points show individual measurements on specific days and are therefore only joined up for consecutive days.

3.4 Discussion

The results of the adult CF patients presented in this chapter represent the largest study to date. The data show that the possession of two abnormal structural *MBL-2* alleles (O/O) is associated with significantly reduced lung function.

Table 3.4 summarises the 7 studies now published on this topic, including the data from this chapter (Davies et al., 2004;Davies et al., 2002). Six of these have shown that MBL polymorphisms play a modifying role on lung function in CF, the other study (Carlsson et al., 2005) found the effect of MBL only in CF patients colonized with *S. aureus*. Two, (Garred et al., 1999b;Gabolde et al., 1999) (see introduction) were published prior to the commencement, and 4 during completion of this work. In 40 Belgian and 72 Czech CF patients, with a mean age of 13.4 years, Yarden's group demonstrated that those with an MBL genotype A/O or O/O were significantly more likely to have an FEV_1 % predicted value of less than 90% compared to those A/A patients. This remained the case when groups were divided into MBL low producing haplotyes (XA/XA, YA/O, X/AO and O/O) and high producers (YA/YA and YA/XA), (Yarden et al., 2004). It was subsequently also shown in a small cohort of 47 Italian adult CF patients that FEV_1 was significantly reduced in those with at least one MBL variant allele (Trevisiol et al., 2005), and in American CF adults that FEV_1 was significantly reduced in those with at least one MBL variant allele but only in those CF patients homozygous for the ΔF508 mutation (Choi et al., 2006). Lastly, conversely, Carlsson's group found in 112 Sweedish CF patients aged 4-54 that there was no *overall* correlation between MBL deficiency and reduced lung function or need for transplantation although in a subgroup of 27 *Staphlococcus aureus* infected patients this

association was in fact seen (Carlsson et al., 2005). Only one study specifically analysed adults and children separately (Davies et al., 2004).

In the results section of this chapter, data from the two largest and youngest cohorts of children to date are also presented. Neither cohort showed a difference in lung function between the variant allele and wild type groups. This may reflect that MBL reveals itself as a modifyer of CF lung disease only in older patients with increased disease severity. In view of this difference between children and adults, variations between the various studies may in part be explained by the differing mix of children and adults included in their analyses.

MBL variant alleles were however overrepresented in older GOSH children who died or required lung transplantation in line with Garreds original results. One child with a liver transplant was also MBL deficient. Taken together these results lend support to the hypothesis that MBL does indeed play a role in modifying CF lung (and possibly also liver) disease but only in older children and adults with established lung disease.

It can be seen from data presented here, that serum MBL levels were higher in the CF children than in published "healthy" controls where the mean of A/A genotype was 1600ng/ml (Turner, 1996). This could be due to differences in the age of the population or more likely reflects the effect that CF chronic lung disease has on serum MBL. To further investigate the relationship between MBL level and lung function, both the Brompton adult and GOSH paediatric population had lung function analysed according to highest, middle and lowest MBL serum quintiles. Unexpectedly a clear and similar pattern was seen in both adults and children in that those with serum levels in the lower and upper quintiles had reduced lung function compared to those with levels in the middle quintile. Low levels of MBL may be due to low production due to gene polymorphisms, consumption during disease or reportedly in SLE, due to the binding of

MBL autoantibodies (Mok et al., 2004). Having genotype data for these cohorts makes it clear that the lower serum quintile levels were due to low expressing MBL genotypes. Similarly the patients with reduced lung function and MBL serum levels in the upper quintile had wild type alleles. It is unclear if the levels in these patients reflect the *consequence* or the cause, of poor pulmonary status. It is tempting to speculate that as an acute phase protein, these high levels represent a sustained "acute phase response" in those with poor lung function and high expressing genotypes. In support of this hypothesis is data from the serial MBL levels performed and presented in this chapter, where those patients with high expressing genotypes could increase their MBL levels, to many thousand ng/ml, with time.

How MBL is operating in CF is still unclear. Two of the studies (Garred et al., 1999b;Gabolde et al., 1999) suggested that MBL's role in the acquisition of *P.aeruginosa* or *B.cepacia* may be important. However the studies presented here did not show an association between MBL genotype and susceptibility to *P.aeruginosa* or *B. cepacia (*described further in chapter 5) in adults or children. This may suggest that MBL is not primarily responsible for protecting CF patients from infection.

MBL plays a complex and incompletely understood role in the inflammatory response and a biphasic dose response has been demonstrated in an ex vivo model (Jack et al., 2001b). Here the addition of high concentrations of MBL (>6000 ng/ml) to the blood of MBL deficient donors decreased the production of proinflammatory cytokines IL-6, IL-1β and TNF-α by monocytes in response to infection, whereas low concentrations enhanced the production of IL-6 and IL-1β. Cystic fibrosis is known to be a disease characterised by marked inflammation as well as infection (Armstrong et al., 1997) and it maybe that MBL deficient individuals have a more pronounced deleterious

inflammatory response in certain conditions such as in patients with CF and/or those in intensive care (see chapter 6).

Modifier studies are important in aiding the understanding of disease pathogenesis and may influence the development of novel treatments. They must however only be taken seriously if repeated studies with large numbers of patients show the same results. The work described in this chapter lends substantial weight to the hypothesis that MBL plays a modifying role CF lung disease. From this work, and other studies (Table 3.4) this seems to occur in older children and adults but not in younger children. This may be due to genetic modifiers of disease being masked by better care nowadays resulting in almost normal lung function in early childhood thus the modifying role of MBL does not become apparent until later in life.

If MBL does play a role in the modulation of inflammation in lung disease, as well as its known effects relating to respiratory infection (Koch et al., 2001), exactly how it exerts its effect in the lung is also unknown but it seems probable that getting to the airway surface is important. The study described in the next chapter was therefore designed to answer the question "*Is MBL present at the airway surface?*"

Study authors, country & publication year	No of CF patients	Mean/ median age in years	MBL groups compared	Association of MBL genotype and lung function	Association of MBL genotype and bacterial pathogens
Garred et al Denmark 1999	149	16.2 (median)	A/A vs A/O + O/O	LFT's significantly ↓ & reduced survival in A/O + O/O	↑ B.cepacia and trend towards younger age P.a in A/O + O/O
Gabolde et al France 1999	22	19.9 (mean)	A/A vs O/O	LFT's significantly ↓ in O/O	Trend towards ↑ P.a carriage in O/O
Yarden et al Belgium & Czech,2004	112	13.4 (mean)	A/A vs A/O + O/O	Significantly more A/O + O/O had FEV_1 <90%	No association with age of acquisition P.a
Davies et al UK 2004	298 adults, 260 children	29.7 (mean) 9.7 (mean)	A/A vs A/O vs O/O	LFT's & O2 saturation significantly ↓ in O/O adults only. No difference in children	No association with age of acquisition P.a or B.cepacia.
Carlsson et al Denmark, 2005	112	20.5 (median)	A/A+YA/O vs XA/O+O/O	No overall association but in 27 patients with *S.aureus*, XA/O & O/O associated with ↓ LFT's	↑ P.a in MBL *sufficient* pts
Trevisiol et al Italy, 2005	47	18.6 (median)	A/A vs A/O + O/O	FEV_1 significantly ↓ in A/O + O/O	Significantly earlier age of onset of P.a in A/O + O/O
Choi et al USA 2006	135	33.6 (mean)	A/A vs A/O + O/O	FEV_1 significantly ↓ in A/O + O/O in those δF508/δF508 only	No association with *P.a*

Table 3.4 Results of all published studies in the field of MBL and cystic fibrosis.

Six of the 7 studies found an association between MBL polymorphisms and reduced lung function in CF patients. The other study (Carlsson et al, 2005) also found the association but only in patients colonized with *S.aureus*. Differences are seen as to whether the effect is seen just in those homozygous, or heterozygous as well, for an MBL polymorphism. Only one study (Trevisiol et al 2005) found a significant association with MBL polymorphisms and P.a infection. Data from this chapter is included (Davies et al, 2004). LFT's = lung function tests, *P.a.= P.aeruginosa.*

CHAPTER 4

Mannose-binding lectin and airway surface fluid

4.1 Introduction

In chapter 3, data were presented that showed MBL genotype was associated with disease phenotype in CF. This concurred with a number of other studies outlined in the chapter (Davies et al., 2004;Garred et al., 1999b;Gabolde et al., 1999;Yarden et al., 2004). MBL is also associated with non-CF lung disease, specifically MBL insufficiency is associated with acute respiratory tract infections and pneumococcal disease, (Koch et al., 2001;Cedzynski et al., 2004;Roy et al., 2002) (see section 1.6.1.1). Taken together it would appear that MBL is important in lung infection/inflammation, however it is unclear *how* MBL exerts its effects in the lung.

The respiratory tract is continually challenged by infective, inflammatory and allergenic agents against which it exhibits an array of innate and adaptive defence mechanisms. The innate immune system in the lung consists of structural (e.g. mucociliary escalator) and cellular (e.g. alveolar macrophage) components together with excreted proteins such as defensins, lysozymes, lactoferrin and the hydrophilic surfactant proteins (SP) A and D. The latter surfactant proteins, produced by alveolar epithelial cells, have many structural and functional similarities with MBL and are also members of the collectin family. Specifically all three proteins are macromolecules which contain N-terminal collagen-like regions involved in trimerisation and C-type lectin domains (Sastry and Ezekowitz, 1993) (see chapter 1, section 1.2). SP A and D, like MBL, bind surface carbohydrates on pathogens, enhance phagocytosis and can modify cellular activation and the inflammatory response (Clark et al., 2000;Crouch, 1998;van Iwaarden et al.,

1990;Malhotra et al., 1994a). All three are encoded by genes located on the long arm of chromosome 10, between 10q21-24, suggesting a common ancestral gene (Hansen and Holmskov, 1998). Although primarily considered an airway surface protein, SP-D mRNA and protein has been identified in human mucosal surfaces as diverse as brain, heart, gut, kidney and skin implying that this protein and its function is not restricted to the lung (Madsen et al., 2000). Similarly in mice, extra-hepatic expression of MBL has now been demonstrated in the lung, kidney and small intestine and from cells of the monocyte/macrophage lineage (Uemura et al., 2002;Wagner et al., 2003). Clinical evidence of MBLs involvement in lung disease together with MBL's similarities to SP-A and D raises the possibility that MBL might be active on the airway surface in the presence of disease. If this is true then it seems plausible that MBL, like the surfactant proteins, may be capable of contributing to host defense and/or the modulation of inflammation in the lung.

The aim of the work described in this chapter was therefore to determine whether MBL is present on the airway surface in either health or disease. To answer this question, BAL fluid was analysed for the presence of MBL from a large group of children with and without lung disease. One group of children with acute, not chronic, disease was specifically recruited to ensure that any effects of chronic disease, such as potential MBL breakdown, could be ascertained. Chronic diseases included cystic fibrosis, recurrent lower respiratory tract infections (> 2 LRTI with radiological change) and primary ciliary dyskinesia. The latter is a rare autosomal recessive condition, caused by "dysmotile" cilia and characterized by recurrent lower respiratory tract infections, sinus disease and glue ear. To assess the extent of the inflammatory process in the lung, a number of markers could have been looked at. However the patients with chronic disease were recruited from the Royal Brompton Hospital as part of another study and

therefore only a single assay, human neutrophil elastase concentration, could be performed. This proteolytic enzyme, derived from inflammatory cells has been shown to correlate with severity of inflammation (Lengas et al., 1994) and airway obstruction in CF (Meyer and Zimmerman, 1993).

4.2 Methods

4.2.1 Patient Population

Children undergoing an acute or elective bronchoalveolar lavage (BAL) were recruited during a 6 month period in 2002 at Great Ormond Street Hospital by myself and a 15 month period in 2003-4 at the Royal Brompton Hospital by Dr Tom Hilliard (Respiratory Research Fellow). They were divided prospectively into the following 6 groups:

1) Acute Pneumonia or pneumonitis (PP): These children were all ventilated on the Paediatric intensive care unit (PICU) at Great Ormond Street Hospital, where a diagnosis of pneumonia or pneumonitis was made on the basis of radiological changes with a consistent history. Two additional patients with empyema, who did not undergo bronchoscopy, had pleural fluid analysed.

2) Cystic fibrosis: These children had been diagnosed by sweat test and/or genotyping and were undergoing bronchoscopy and BAL either as a routine investigation at the time of diagnosis, for lack of response to antibiotic treatment or for research purposes at the time of a general anaesthetic for a surgical procedure.

3) Primary ciliary dyskinesia (PCD): This had been diagnosed on nasal brushing samples and these children were undergoing elective bronchoscopy for microbiological surveillance.

4) Recurrent lower respiratory tract infections (RLRTIs): These children had at least 2 lower respiratory tract infections (LRTIs) and were undergoing elective bronchoscopy during a period of clinical stability (i.e. after resolution of acute illness) as part of a respiratory work-up.

5) Acute non-infective conditions (e.g. postoperative, trauma): these ventilated children were undergoing non-bronchoscopic BAL for failure to extubate and/or to exclude infection. These patients all subsequently had a non-infective diagnosis made such as veno-occlusive disease, or failure to extubate due to CNS trauma. In addition they all had negative microscopy for organisms, negative bacterial, fungal and virological cultures and negative PCRs for a wide panel of respiratory viruses. These patients therefore acted as part of a "control" group.

6) Upper airway problems: These patients underwent bronchoscopy for the investigation of upper airway (UA) structural problems. Ethical approval had previously been obtained to perform BAL on these patients who also formed an important "control" group. In none were any adverse effects of the BAL seen.

For most analyses of results these groups were analysed independently but for the purposes of discussion, patients from group 1 are referred to as having acute disease, groups 2-4 having chronic disease and groups 5 & 6 being the "controls".

Patient demographic and microbiological/virological data were obtained from the routine laboratories at each institution or from the referring hospital. Local Research Ethics Committee approval was obtained from Great Ormond Street Hospital and The Royal Brompton, Harefield and NHLI Trust. All parents or carers gave informed consent and assent was obtained from children where appropriate.

4.2.2 Bronchoalveolar lavage collection and sample processing

BAL was performed with 1-3 aliquots of 1ml/kg (maximum 30 mls) sterile 0.9% saline in either the right middle lobe (bronchoscopic) or aimed at a region of radiographic change (non-bronchoscopic). The latter was done by positioning of the child's head and

neck to attempt lavage of either right or left side if possible. 15/17 patients on the intensive care unit had a non-bronchoscopic BAL performed by a senior physiotherapist. The remaining two PICU patients and all the RBH patients had a bronchoscopic BAL performed by a Paediatric Respiratory Consultant. BAL fluid aliquots were pooled, and provided sufficient was available for required clinical investigations, a sample was retained for research. Samples were immediately placed on ice and spun at 400g for 10 minutes at 4°C. The supernatant was removed and stored in aliquots at -80°C until analysis.

4.2.3 Empyema and sputum collection and sample processing

Two samples of empyema (one from a patient on PICU, the other on the respiratory ward) and two samples of expectorated sputum (from two adolescent male CF patients, both with severe bronchiectasis and *P.aeruginosa* infection) were collected into specimen pots on ice. All samples were processed in the same manner as the BAL samples in section 4.2.2. and MBL levels subsequently determined by ELISA.

4.2.4 Influence of Protease Inhibitors on Resultant MBL levels

In order to determine whether protease inhibition, post BAL collection, was necessary to prevent potential MBL degradation, the supernatant from 14 patients was divided and treated with or without a cocktail of protease inhibitors (Complete Mini, Roche Diagnostics GmbH, Mannheim, Germany). These were added immediately after separating the supernatant in accordance with the manufacturer's instructions. These 14 patients all had a diagnosis of acute pneumonia/pneumonitis or acute non-infective conditions. 7 samples had no MBL detected either with or without PI and no sample had MBL detected only in the presence of PI. In the seven with MBL present there was no

significant difference in the MBL levels with and without PI. Therefore, in the rest of the study, samples were not treated with PI.

4.2.5 Blood samples for serum MBL levels & MBL genotyping

1-2 mls of blood were spun, separated and the serum stored in aliquots at -80°C until analyzed. In the PICU patients and some CF patients 1-2 mls of whole blood in EDTA were also taken for MBL genotyping.

4.2.6 SDS Polyacrylamide gel-electrophoresis and western blotting

SDS Polyacrylamide gel-electrophoresis and western blotting were performed to determine whether MBL was present in BAL fluid. 10 % polyacrylamide gels were cast using Protogel reagents (National Diagnostics, Hull, UK) in gel casting units (Amersham Pharmacia Biotech, Bucks, UK). BAL samples, together with a positive (serum of YAYA donor, or MBL "spike" of 5 μg/ml) and negative (bovine serum albumin) control, were diluted with an equal volume of 2 x sample buffer containing 4 % 2-mercaptoethanol (freshly prepared), and heated at 100°C for 5 min. Samples were loaded into the gel and electrophoresed at 120 V for 1 hr at room temperature alongside a rainbow colour marker and biotinylated broad range size standard (Bio-Rad, Hemel Hempstead, UK). To visualise protein bands gels were stained with Coomassie Blue stain.

For Western blots, proteins were transferred onto Hybond P nitrocellulose membranes (Pharmacia Biotech, Bucks, UK) using a semi-dry transblotter (Trans-Blot, Bio-Rad, Hemel Hempstead, UK). Gels were carefully applied to the nitrocellulose membranes and sandwiched between 6 pieces of filter paper which, along with the membranes, had been pre-soaked in Transbuffer. Transfer was performed by electrophoresis at 12 V for 40 min ensuring the colour marker was present on the membrane. Transfer was also

checked by staining with Ponceau S stain. The membranes were then blocked in "block solution" of TBS-Tween containing 0.2% I-block™ (Applied Biosystems, CA, USA) for 1 hr at room temperature on a shaking platform to prevent binding of any free proteins. Membranes were washed three times for ten minutes in TBS- Tween before being probed by biotinylated anti-MBL 1/5000 in block solution for 3 hours at room temperature on a roller. After further washing detection was performed with Streptavidin-Horseradish peroxidase (Serotec Ltd, Oxford, UK) 1/1000 in block solution for 1 hour at room temperature on a roller. After further washing blots were developed using ECL chemiluminescent kit (Amersham Int, Little Chalfont, UK) according to manufacturers instructions. Proteins were visualised by exposure to X-ray film initially for 15 seconds with further longer exposures determined by the results of the initial exposure.

The technique used here was a standard procedure optimised by myself to achieve better visualization of protein bands. Optimisation involved using different concentrations of primary antibody (1/1000-1/10 000), Streptavidin-HRP (1/000-1/10 000) and skimmed milk (2-5%). The use of a highly purified casein based blocking reagent, I-block™ 0.2% (Applied Biosystems, CA, USA) was finally used as milk contains avidin, which binds biotin, so casein based blocking solutions help reduce background.

4.2.7 MBL protein levels by ELISA

MBL levels in BAL fluid, serum and empyema fluid were determined by a symmetrical sandwich ELISA (Antibody Shop, Copenhagen, Denmark). Serum was diluted to 1/50-1/200, whereas BAL was used neat or 1/2 dilution, with results being corrected for the dilution factor. The standard curve was obtained using the ELISA kit standards ranging from 0.5-40 ng/ml. Results higher than 6500 ng/ml (serum samples) were repeated with further dilutions as appropriate. Levels below 4ng/ml were counted as zero. Samples

were anonymised ensuring "blinding" to patient diagnoses until the results were analysed.

4.2.8 Neutrophil elastase

Neutrophil elastase (NE) was measured in BAL with a colorimetric assay based on NE cleaving nitroanilide from a substrate, N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide according to manufacturer's instructions (Sigma Aldrich, St Louis, USA). 10µl sample volumes were used and levels were expressed as milliunits (mu)/ml. 1mu activity was the lowest standard tested and the range of the assay was 20 - 5000 mu/ml, therefore any value below 20 mu/ml was taken as zero. This assay was performed by Dr Tom Hilliard, Clinical Research Fellow, Royal Brompton Hospital.

4.2.9 Statistical analysis

Differences between multiple groups in both studies were assessed using Kruskal-Wallis (KW), Mann Whitney (MW), Chi squared or Wilcoxon signed rank tests as appropriate. Spearman and Pearson's correlations were performed where appropriate. As the majority of the data were non-parametrically distributed, results are presented as median (range). Analysis was performed using GraphPad Prism version 3.0 for Windows, GraphPad Software, San Diego California USA, and SPSS for windows v.9.

4.3 Results

4.3.1 BAL Patient Demographics

85 children were recruited to this study. Patient demographics and diagnoses are shown in Table 4.1. 17 patients had urgent BAL performed whilst ventilated on PICU. The remaining 68 BALs were performed electively. Empyema fluid (n=2) and CF sputum (n=2) was also evaluated in 4 further children from GOSH. Controls included 3 children with upper airway obstruction and 4 with acute non infective "other" diagnoses a) one with severe asthma, b) one with pulmonary veno-occlusive disease, and c) two children who failed extubation, one after a severe head injury and one with a non-pulmonary malignancy. 40/85 (47%) were male and the median age was 5.7 years (1 month -16.8 years). There were no significant differences in the mean ages (p=0.25, KW) or gender proportions (p=0.13, chi-squared) between disease groups.

Diagnostic group (no.)	**Male:female** (% male)	**Age** Median (range)
Acute pneumonia/ pneumonitis (13)	9 : 4 (69)	4 (0.1-16.3)
Cystic fibrosis (37)	13 : 24 (35)	7.7 (0.3-16.8)
PCD (6)	2 : 4 (33)	9.5 (5.7-14.8)
RLRTI (22)	11 : 11 (50)	5.3 (0.9-15.2)
Acute "other" (4)	2 : 2 (50)	8 (0.2-15.1)
Upper airway (3)	3 : 0 (100)	7.2 (5.8-7.5)
P value	**p=0.13 ns** **Chi squared**	**p=0.25 ns** **KW**

Table 4.1 Demographic details of 85 patients who had BAL performed.

There was no statistically significant difference in age or sex between the groups.

PCD=primary ciliary dyskinesia; RLRTI= recurrent lower respiratory tract infections.

4.3.2 Relationship of MBL haplotype to MBL serum levels

33 patients had MBL genotypes performed, including all those with acute pneumonia/pneumonitis and about half of those with CF or an acute non infective illness. In these groups approximately 2/3 had a wild type genotype (A/A) and 1/3 were heterozygous for a mutation (A/O). None of these had the O/O genotype. (A/A:A/O – PP 69%: 31%, CF 72%:28%, Acute other n=2, 50%:50%). In the groups who had genotypes performed the observed distribution is not significantly different from that expected in UK published series (Mead et al., 1997). In 78 children serum was available for determination of MBL levels and 27 of these also had whole blood available for MBL genotyping. None of these 27 patients possessed the O/O genotype, hence comparisons were done between two groups. As previously published (Fidler et al., 2004;Davies et al., 2002;Garred et al., 1999b), median serum MBL levels were significantly different between both genotypes (Fig 4.1 A, p=0.0002, Mann-Whitney) and haplotypes (p=0.0063 Kruskal-Wallis, Fig 4.1 B). Levels were not age or sex dependent.

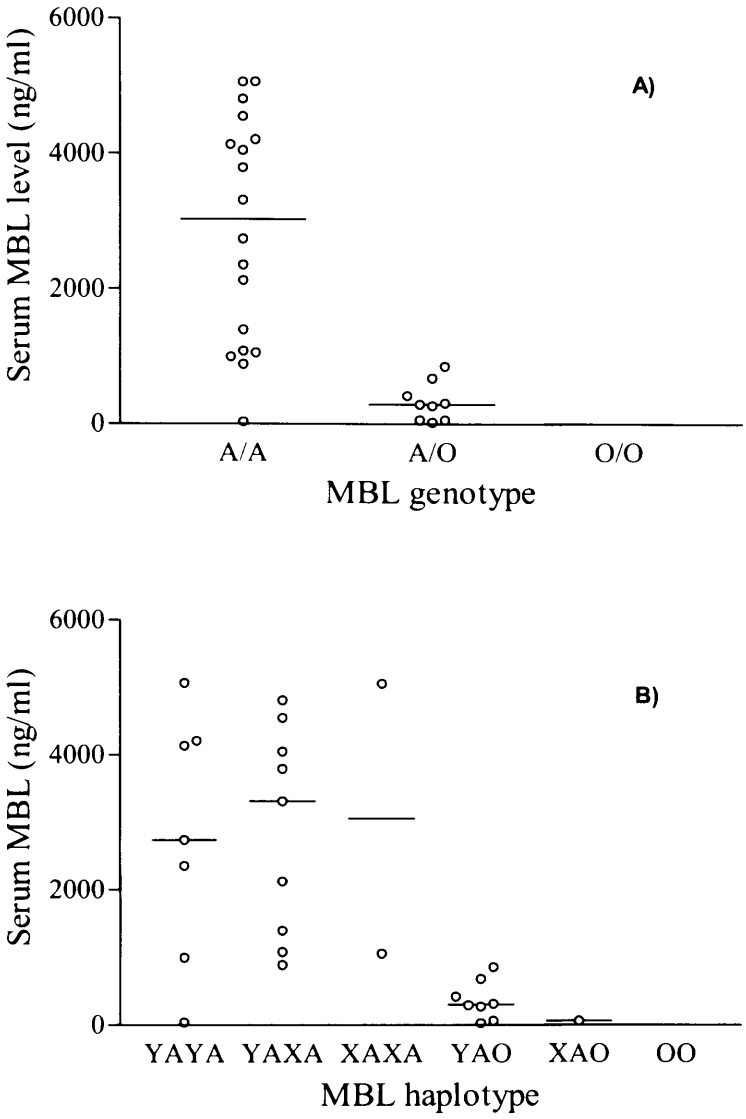


Fig 4.1 Relationship between MBL serum levels and MBL haplotypes. Median MBL levels are significantly different between **A)** MBL genotypes and **B)** haplotypes in 27 patients in whom both were available (p=0.0002 Mann Whitney & p=0.0063 Kruskal-Wallis respectively). Small numbers in the XAXA group (n=2) has resulted in median MBL levels being higher than usually seen. There are no patients in this series with the O/O genotype.

4.3.3 Western Blots

Demonstration of MBL in BAL fluid by western blotting was technically difficult due to the viscosity of the lavage fluid. After a period of optimization it was possible to demonstrate that MBL could be seen in BAL fluid and an example of one Western blot is shown in Fig 4.2. However, although Western blotting could have answered the question of whether or not MBL was found in BAL fluid, it did not provide quantitative data that could be used for comparison between groups. In addition sample volumes were limited and it was a more time consuming technique than originally anticipated. Thus an MBL ELISA kit (Antibody Shop, Copenhagen, Denmark) was subsequently used for all the results shown in this chapter.

4.3.4 Influence of Protease Inhibitors on Resultant MBL levels

No differences were seen in the subgroup of acute samples (n=14), measured by ELISA, with or without the addition of protease inhibitors (Fig 4.3; mean (SEM) values: +PI 15.4(4.9) ng/ml; -PI 17.4(5.7) ng/ml; paired t-test p=0.4) and therefore all results reported now in this chapter are from untreated BAL. This probably reflects the fact that if any protease degradation of MBL occurred, it did so in the lung before BAL collection. It is likely that the rapid placement of BAL samples on ice and immediate processing limited further MBL breakdown.

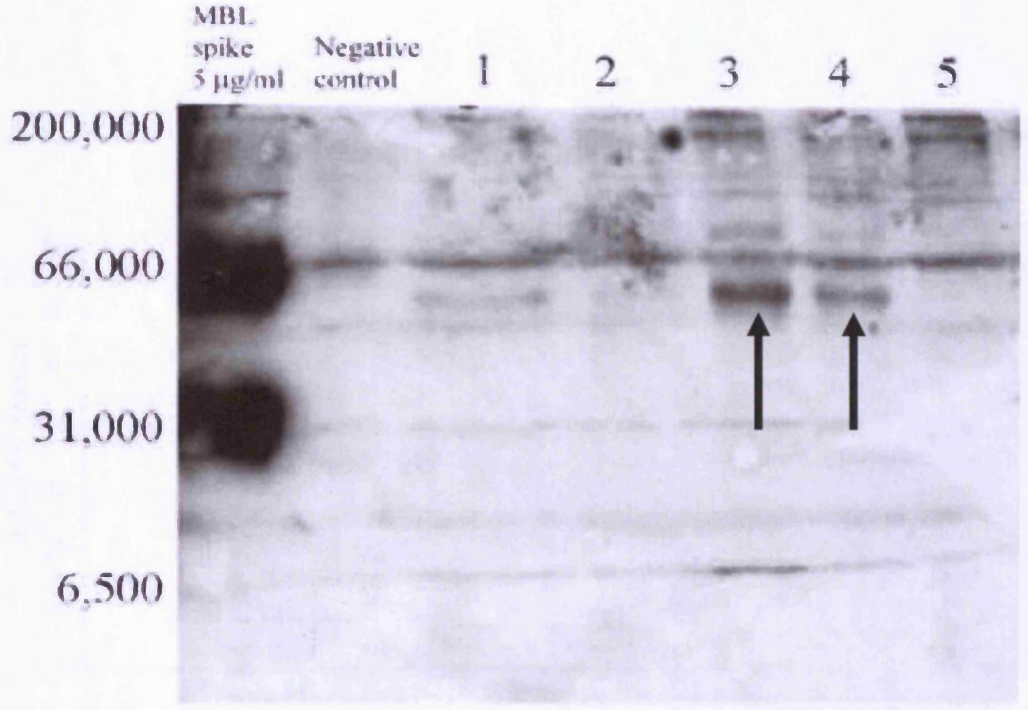


Fig 4.2 Western blot demonstrating the presence of MBL in BAL fluid

Lanes 1-5 show patient BAL samples (diluted in sample buffer containing 4% 2-mercaptoethanol and denatured at 100°C for 5 min). Lanes 1 & 2, 3 & 4 and 5 are three different patients without (1, 3 & 5) and with (2 & 4) the addition of protease inhibitors. A positive control of high concentration MBL (5µg/ml) and a negative control of bovine serum albumin are also demonstrated. Lanes 3 and 4 clearly show a specific band (marked by arrows) for MBL (probed by biotinylated anti-MBL) at a size expected for denatured MBL (approximately 64,000 Kd). A non specific band is seen in all lanes. A biotinylated broad range size standard was included but omitted here for clarity.

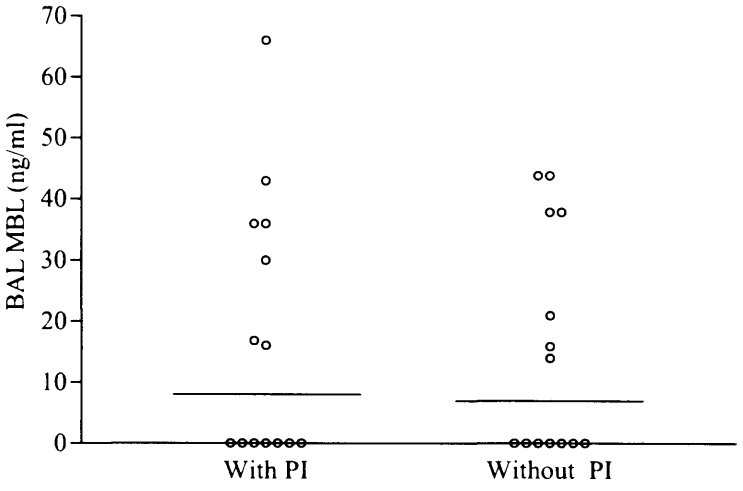


Fig 4.3 Influence of protease inhibitors on MBL levels. No differences were seen in the subgroup of samples (n=14), measured by ELISA, with or without the addition of protease inhibitors (median (IQR) +PI 8 (0-36)ng/ml and -PI 7 (0-38) ng/ml respectively, p= 0.47, Wilcoxon signed rank test) The values of every sample pair +/- PI were significantly similar, (effectiveness of pairing p=<0.0001, Spearman rest 0.93).

4.3.5 MBL levels in BAL, empyema fluid and sputum

MBL was detected in 17 BAL samples overall (20%) and could be found in a proportion from each of the groups with pulmonary disease but in no child without lower respiratory disease (Figure 4.4). The acute pneumonia/pneumonitis group had a significantly greater proportion of patients with MBL present in the BAL ($p=0.0018$, $\chi 2$ test) and median levels that were significantly higher than those in any of the other groups (Figure 4.5; KW $p<0.001$). For those subjects with MBL detectable in the BAL, there was an unexpectedly significant negative correlation with serum MBL measured simultaneously (Figure 4.6; Pearson $r = -0.72$, $p=0.002$). MBL was present in pleural fluid from both patients with empyema (empyema levels 38 ng/ml and 1178 ng/ml, genotypes XAO and YAXA respectively) but absent from both CF patients' sputum (Table 4.2).

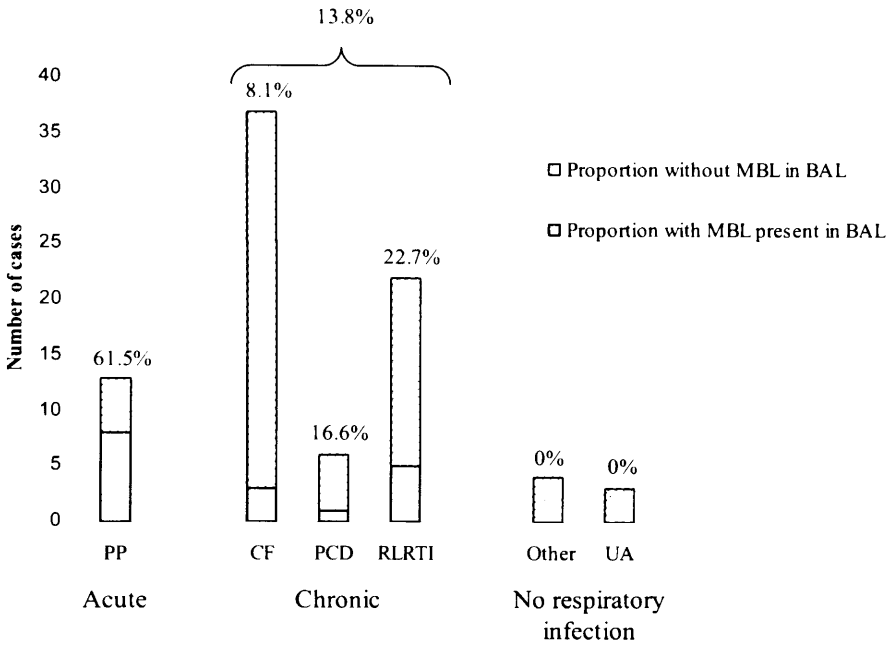


Fig 4.4 Proportions of patients with MBL in bronchoalveolar lavage. Proportions of patients in the disease groups with MBL detectable in BAL (grey area and % figures) differed significantly ($p<0.01$), highest levels being observed in the acute pneumonia/ pneumonitis group. No MBL was present in any of the 7 patients with no respiratory disease.

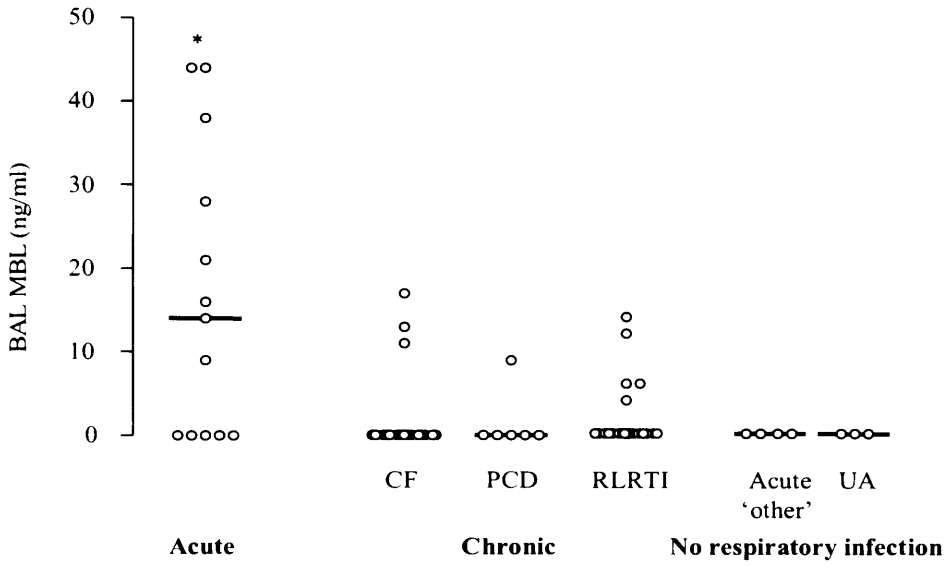


Fig 4.5 BAL MBL levels and relationship to disease group. Median level of MBL was significantly higher in patients with acute pneumonia/ pneumonitis (PP) infection than in the other groups (KW $p<0.001$; * $p<0.001$ vs chronic respiratory disease, $p<0.05$ vs no respiratory infection).

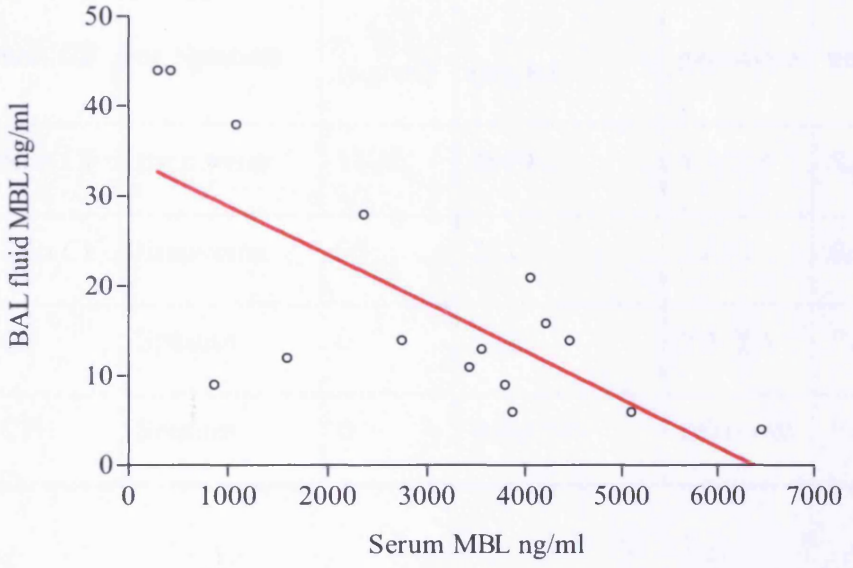


Fig 4.6 Relationship between MBL in serum and in BAL fluid.

In 16/17 children with MBL present in BAL fluid, serum samples were also available for determination of MBL levels. A significant negative correlation between serum and BAL MBL levels is apparent (Pearson r = -0.72, p=0.002).

Patient No	CF or non CF	Empyema or Sputum	MBL (ng/ml)	Serum MBL (ng/ml)	MBL genotype	Organism cultured
1	Non CF	Empyema	1178	4079	YA/XA	*S.aureus*
2	Non CF	Empyema	38	271	XA/O	*Bacteroides*
3	CF	Sputum	0	7222	YA/YA	*P.aeruginosa*
4	CF	Sputum	0	unknown	unknown	*P.aeruginosa*

Table 4.2

Relationship of MBL in empyema fluid or sputum to serum MBL levels, MBL genotype and organism cultured. Both patients with empyema had MBL detected in their empyema aspirates. Neither of the children with CF, *P.aeruginosa* infection and chronic lung disease had MBL detected by ELISA in their sputum.

4.3.6 Microbiology

Overall a causative organism was found in 30/85 children (35%) (PP:6, CF:12, PCD:3, RLRTI:9, acute "other":0, UA:0). Many children had received antimicrobial treatment prior to relevant bacterial and virological cultures being obtained and therefore this detection rate was probably falsely low. 29 strains of pathogenic bacteria were isolated from BAL fluid from 26 (30%) children (*P. aeruginosa* n=7; *H. influenzae* n=7; *S. pneumoniae* n=6; *S. aureus* n=6; *S. maltophilia* n=2; *B. catarhalis* n=1). Two children in the acute PP group had viruses isolated from the BAL: *Epstein Barr* virus, and *Influenza A*. Two further children in the PP group had organisms isolated from other sites, one with varicella pneumonitis and disseminated disease (isolated from skin), the other with Group A streptococcal pneumonia and toxic shock (isolated from blood culture). None of the children assigned to the 'acute non-infective' or upper airway groups had positive cultures. Due to small numbers in some of the groups, the differences in the proportions of patients from each group yielding a positive culture did not reach significance (PP: 46.2%, CF:32.4%, PCD:50%, RLRTI:40.9%, acute "other":0%, UA:0%; p=0.36), although children with airway disease were significantly more likely than those in the control groups to have a positive culture (38% vs 0%, p=0.041). Neither those in whom bacteria were isolated, nor the combined bacteria/ virus isolated group had a greater likelihood of MBL being present in their BAL nor higher median BAL MBL levels. The two empyema fluid samples grew *Bacteroides fragilis* and *S. aureus* (empyema MBL levels 38 ng/ml and 1178 ng/ml respectively).

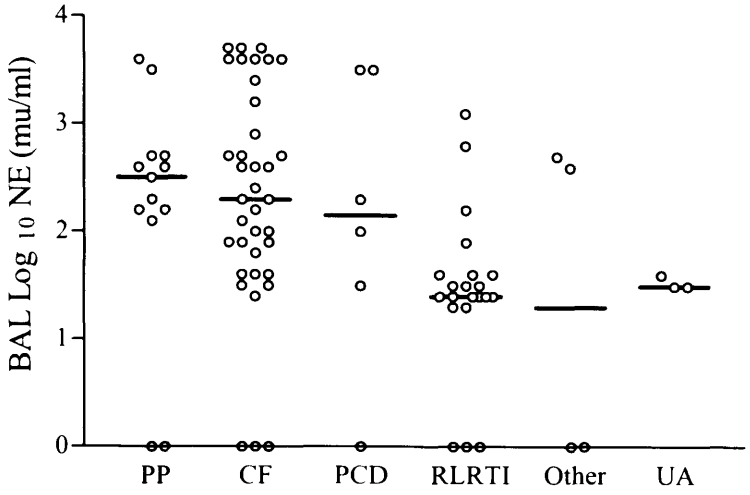
These data, together with MBL in BAL data is summarised in table 4.3.

Diagnostic group (n)	**No (%) with MBL detected in BAL**	**BAL MBL level ng/ml; median (range)**	No (%) with positive microbiology/ virology (n=30)
Acute pneumonia/ pneumonitis (13)	8 (61.5)	14 (0-44)	**6 (46.2)**
Cystic fibrosis (37)	3 (8.1)	0 (0-17)	**12 (32.4)**
PCD (6)	1 (16.6)	0 (0-9)	**3 (50)**
RLRTI (22)	5 (22.7)	0 (0-14)	**9 (40.9)**
Acute "other" (4)	0 (0)	0 (0-0)	**0 (0)**
Upper airway (3)	0 (0)	0 (0-0)	**0 (0)**
P value	p=0.0018 Chi squared	p= 0.0005 KW	**p=0.36 ns Chi squared**

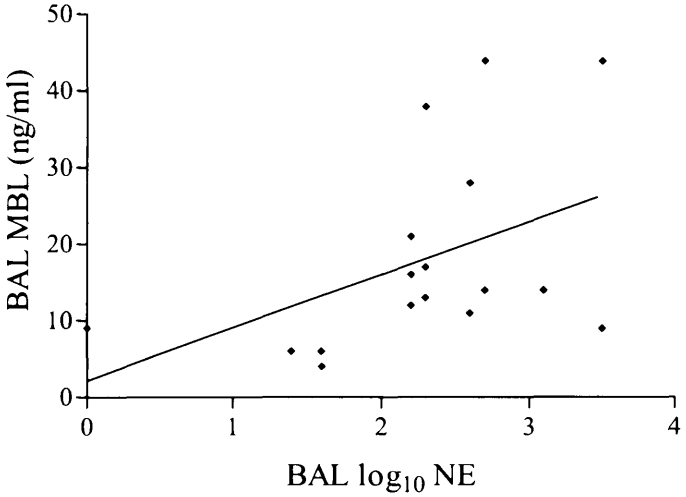
Table 4.3 Details of 85 patients who had BAL performed. Significantly more patients in the acute pneumonia/pneumonitis group had MBL detected in BAL fluid and at significantly higher levels than other groups. There was no statistically significant difference in age, sex or those with a positive culture of an organism between the groups. PCD=primary ciliary dyskinesia; RLRTI= recurrent lower respiratory tract infections.

4.3.7 Neutrophil elastase (NE)

Levels of airway inflammation as determined by neutrophil elastase (NE) differed significantly between groups ($p<0.001$ KW, Fig 4.7A). Median levels were high in the acute PP (285[0-4044] mu/ml), CF (211[0-5005] mu/ml) and PCD (134[0-3502] mu/ml) groups, with no differences between them. Significantly lower levels were observed in both the RLRTI (27.7[0-1297] mu/ml; $p<0.01$ vs acute PP and CF) and no respiratory infection (control) groups (32.3[0-579.8] mu/ml; $p<0.05$ vs CF). For the group as a whole, levels of BAL MBL did not correlate significantly with NE, although for the subgroup with detectable MBL (n=17), there was a positive correlation (Fig 4.7B, $r=0.52$; $p<0.05$). This relationship was held up in the acute pneumonia/pneumonitis group with detectable MBL (n=8; $r=0.73$; $p<0.05$) but numbers were not sufficient to further analyse this relationship in other disease subgroups.



A)



B)

Fig 4.7 Relationship of BAL neutrophil elastase levels to clinical groups and BAL MBL levels **A)** NE levels were significantly higher (log fold) in the PP, CF and PCD groups compared to RLRTI and controls (p=0.001, Kruskal-Wallis). **B)** In the cohort with MBL detected in the BAL (n=17), there was a significant positive correlation of BAL MBL with concentration of neutrophil elastase (r=0.52; p=0.027, Spearman).

(PP=acute pneumonia/pneumonitis, controls=acute "other" and upper airway patients).

4.4 Discussion:

The work described in this chapter is the first large human study to attempt to measure MBL at the airway surface and demonstrates that, whilst the protein is undetectable in normal health, it is present in a variety of lung disease states in children.

This led to the hypothesis that some of the links reported of MBL-deficiency and lung disease might relate to the fact that MBL is able to play a role in local host defence. Children with acute pneumonia/ pneumonitis were most likely to have MBL detected in the BAL and had the highest concentrations; intermediate levels were observed in more chronic disease states and the protein was not detectable in any child from the control groups. When present, concentrations of MBL correlated with those of neutrophil elastase, a marker of local inflammation.

Low levels or absence of MBL in chronic disease may be due to reduced leakage or secretion into the airways or increased breakdown of the protein once there. The latter would seem more likely in the patients with CF and PCD for two reasons. Firstly the degree of inflammation, as assessed by the surrogate marker of inflammation neutrophil elastase, is likely to correlate with epithelial leak and was similar in both the acute and chronic CF and PCD groups. It is known that these groups have a marked infective/inflammatory component most of the time and therefore at the time of BAL. In contrast almost all the children with recurrent lower respiratory tract infections were relatively well at the time of the BAL which may explain the finding of near control levels of NE in these patients. This subgroup may well therefore have had reduced leakage or secretion of MBL into the airway compared to those with acute pneumonia/pneumonitis. Secondly, both CF and PCD BAL contain high levels of proteolytic enzymes (Armstrong et al., 1997), which have been shown to degrade the

structurally similar proteins SP-A and D (Griese et al., 2004;Rubio et al., 2004). In further support of protein breakdown, Garred *et al* reported rapid disappearance of MBL when normal human serum was mixed with CF sputum (Garred et al., 1999b) and in the two CF sputum samples tested here no MBL was detected.

Although several publications have reported increased susceptibility to, or severity of, respiratory disease in patients with MBL-deficiency (Koch et al., 2001;Cedzynski et al., 2004;Roy et al., 2002;Garred et al., 1999b;Gabolde et al., 1999;Davies et al., 2004;Carlsson et al., 2005) the question of whether or not MBL can reach, and subsequently act at the airway surface has not been extensively explored. One previous murine study reported that MBL was absent in the bronchoalveolar lavage (BAL) fluid of healthy mice, but detectable after intratracheal instillation of influenza A virus (Reading et al., 1997). In parallel to the human cohort described in this chapter, Dr Jane Davies and colleagues developed a system to explore acute pulmonary infection in a murine model, and demonstrated that MBL was undetectable in BAL fluid in the healthy airway or after instillation of PBS, but appeared as early as 4 hours after infection with *B. cenocepacia* (personal communication). No human study had been published at the time of undertaking this work although subsequently Gomi *et al* have also shown, in a small adult cohort, that MBL can be found in BAL fluid in the presence of respiratory disease (n=5) but not in healthy controls (n=5) (Gomi et al., 2004). Levels determined by ELISA (11-78 ng/ml) were found to be very similar to those reported here.

Although the work described here demonstrates an important finding, namely that MBL can be detected in BAL fluid in disease but not health, there are a number of limitations to this work. This is inevitable, since lower airway sampling can only ethically be performed in children when indicated clinically or at the time of another procedure, and

cannot be scheduled electively for research. Firstly the numbers of patients in the control groups were small, and had a variety of different conditions. These small numbers reduced power for some of the statistical comparisons. However, obtaining lavage samples from healthy paediatric lungs is difficult, both logistically and ethically and this is a common limitation of such studies. Secondly the study was not designed to fully explore the mechanisms by which MBL appears at the airway surface. These include passive leak through damaged epithelia, active secretion, local production or a combination of these. With levels of BAL MBL correlating with inflammation, as evidenced by neutrophil elastase, passive leak may be involved although local production has also been inferred by finding MBL-A mRNA in the mouse lung (Uemura et al., 2002). Determination of expression levels in respiratory epithelial cells in the context of disease and health could aid further understanding of this. In addition only MBL and NE were measured in bronchoalveolar lavage fluid and maybe the measurement of further inflammatory markers (such as IL-8), MBL breakdown products and the ratio of total BAL:serum protein levels may have helped give an insight into the underlying pathophysiology of the appearance of MBL in the lung.

To try and investigate the possible mechanism further, BAL MBL levels were correlated with serum MBL levels and unexpectedly there was a significant inverse relationship between them. In fact the 2 patients with the highest MBL levels in BAL fluid (44 & 44 ng/ml) were 2 children with acute pneumonia/pneumonitis who had the lowest serum MBL levels (294 & 422 ng/ml). If MBL level in BAL fluid merely reflects passive leakage from high serum levels then a positive correlation would have been expected. As the converse was seen, active secretion could be involved as well or if passive leak is the mechanism it must also be related to disease severity and damage to the integrity of the epithelium. One possibility is that more severe disease may be

seen in those with lowest MBL serum levels thus accounting for the inverse relationship seen here. Unfortunately numbers here are too small to correlate MBL levels in BAL and serum with paediatric disease severity scores.

It is also unclear whether the oligomeric structure of the MBL found in BAL from MBL deficient or sufficient patients differs, as it does in the serum (Dean et al., 2005;Lipscombe et al., 1995). Further work with non-reduced, gradient gel electrophoresis of MBL from BAL fluid would be needed to determine this.

Lastly, limitations of this study also include the fact that it was not designed to address the question of whether MBL present in the BAL fluid is actually functional in host defence. However, the data presented here provide two possible pieces of evidence that it is. It has been estimated that MBL levels required for complement activation are likely to be in the region of 300-400 ng/ml (Valdimarsson et al., 1998;Neth et al., 2000), whereas the levels that were detected in these BAL samples reached a maximum of 44 ng/ml. However, based on reports using markers to calculate the dilution of epithelial lining fluid (ELF) by instilled saline during BAL (Restrick et al., 1995), levels in the ELF are likely to be 1-2 logs higher than this, therefore within the range for function. Furthermore, very high levels were measured in the empyema fluid of a patient with *Staphylococcus aureus*, an organism known to be bound by MBL (Neth et al., 2000). Further determination of MBL binding to detected respiratory pathogens found in these BAL's may tease out further mechanisms by which MBL may be operating in the lung.

In addition to MBL's role in the clearance of microbes there is increasing evidence of an immunomodulatory effect of MBL (Jack et al., 2001b;Sprong et al., 2004). It has previously been considered whether this may account for the link between MBL-deficiency and CF disease severity (Davies et al., 2004), as it has been shown that MBL binds very poorly to the major CF respiratory pathogen, *Pseudomonas aeruginosa*

(Davies et al., 2000a). Further work looking at MBL binding to one of the most serious bacteria found in CF lungs, *Burkholderia cepacia*, is presented in the following chapter.

In conclusion, MBL, a protein similar in structure and function to the collectins more conventionally associated with the lung, SP-A and D, may contribute to lung host defence by acting locally at the airway surface.

CHAPTER 5

The Effect of MBL status on the susceptibility of cystic fibrosis patients to *Burkholderia cepacia* complex (Bcc) infection

5.1 Introduction

Burkholderia cepacia complex (Bcc) comprises a group of closely related gram negative bacteria that are important opportunistic pathogens in patients with cystic fibrosis (CF). Infection with these organisms is associated with increased morbidity and mortality (Muhdi et al., 1996;Isles et al., 1984) especially after lung transplantation (De Soyza et al., 2001). In a proportion of patients, a fulminating illness, the "cepacia syndrome" occurs. This is characterised by high temperature, respiratory failure and septicaemia and carries a high mortality (Isles et al., 1984). Clinical concerns also relate to the high transmissibility of some of these organisms and their antibiotic multi-resistance.

Recent taxonomic techniques have revealed that the Bcc comprises a number of distinct genomic species each known as a genomovar, with genomovars I-IX having now been characterised and assigned names (Baldwin et al., 2005;LiPuma, 2005) (Table 5.1). Most clinically relevant species belong to the first 5 genomovars with the most common isolate from CF patients being *B.cenocepacia* (G III) (LiPuma et al., 2001;LiPuma, 2005) of which the Electophoretic type (ET) 12 strain is one of the most virulent & transmissible and has been associated with transatlantic outbreaks. Infection with

different Bcc species affects outcome with mortality being significantly higher in those infected with *B.cenocepacia* than *B. multivorans* (Jones et al., 2004). Predominance of certain strains in CF patients indicates a differential capacity for human infection. This may in part be influenced by differences in host immunity.

It was reported by Garred *et al,* in a small CF cohort, that MBL variant alleles were significantly more frequent in patients with Bcc infection (7 out of 10 patients), (Garred et al., 1999b). This appeared to be confirmed in a preliminary analysis of 13 patients from the Royal Brompton Hospital with Bcc infection in whom an overrepresentation of variant alleles was seen compared to population data from both healthy Caucasian controls and CF patients (8/13, 62%) (Davies et al., 2000b). It was subsequently shown that MBL binds to Bcc clinical isolates and activates complement, to a significantly greater extent than it does to the commonest CF pathogen, Pseudomonas aeruginosa (Davies et al., 2000a).

These data led to the hypotheses that a) MBL-deficient patients are at risk from Bcc infection and b) MBL-deficient patients may be at higher risk of acquiring certain strains of Bcc because the binding of MBL to Bcc may differ between genomovars.

The specific aims of the work described in this chapter were therefore

A) to determine if MBL binding is

(i) present in further Bcc pathogens isolated from patients with CF and

(ii) genomovar related

B) To confirm the hypothesis that patients with MBL variant alleles were predisposed to Bcc infection by looking at a larger cohort of patients than previously studied.

Genomovar designation	**Bcc Species**	**Approximate % ***
I	*B. cepacia*	3
II	*B. multivorans*	40
III	*B. cenocepacia*	45
IV	*B. stabilis*	<1
V	*B. vietnamiensis*	6
VI	*B.dolosa*	4
VII	*B.ambifaria*	1
VIII	*B.anthina*	<1
IX	*B.pyrrocinia*	<1
Indeterminate		1

Table 5.1 Species name and genomovar designation of *Burkholderia cepacia* complex (Bcc) organisms. * Approximate % of infection amongst >1200 Bcc infected CF patients in the USA. Adapted from LiPuma 2005.

5.2 Methods

A) MBL binding to *Burkholderia cepacia* complex

5.2.1 Bacterial Isolates

Twelve freeze dried isolates from the reference panel of Bcc genomovars I-V were obtained (BCCM/LMG Bacterial Collection, Gent, Belgium) (Table 5.2). Thirteen patient sputum samples were obtained from Dr Jane Davies at the RBH. These included 12 adults and one child with Bcc from the CF clinic. Genotypic identification of these organisms was unknown, however pulsed field gel electrophoresis of these isolates showed at least 5 different strains were present (personal communication, Dr Jane Davies).

5.2.2 Preparation and Growth of Organisms

Freeze dried isolates were reconstituted in sterile brain heart broth (Oxoid, Buckinghamshire, UK) transferred to Bcc selective agar (Oxoid, Buckinghamshire, UK) and subcultured twice prior to use as per manufacturer's instructions. Patient samples were incubated on Bcc selective agar at 37°C and subcultured once prior to experimental use. Bacteria were cultured as described by Bridson (The Oxoid manual, 7th ed., Oxoid, Buckinghamshire, United Kingdom). Experiments were commenced 16-18 hours post subculture to obtain organisms in the stationary phase of growth.

5.2.3 Quantification of *Burkolderia cepacia* organisms in Hanks' balanced salt solution ($HBSS^{++}$) at an Optical Density (OD) of 1

Quantification of Bcc bacterial concentration was performed by the method described by Miles & Misra (Miles et al., 1932). Briefly a solution of Bcc bacteria (grown as described in section 5.2.2) in $HBBS^{++}$ was adjusted to an OD of 1 (540nm). 100µl of this solution was taken and serially $\log_{10}$ diluted a further 7 times with $HBBS^{++}$. 4 x

10µl drops of each of the 8 dilutions were plated out on to BCC agar and colonies counted at 24 hours, from which total colony forming units (CFUs)/ml were estimated.

5.2.4 Determination of bacterial growth characteristics

Previous experiments by other researchers on MBL binding to different organisms were conducted approximately 16-18 hours after subculture of the organism, in the stationary phase of growth (Neth et al., 2000;Devyatyarova-Johnson M et al., 2000). To confirm that Bcc organisms were in this phase, one strain, 18870, known on preliminary investigation to be a high MBL binder, was grown in broth and on solid agar at 37 °C. 150µl of Bcc 18870 at OD of 1 (540nm) was added to 2850µl of brain heart broth (1/20 dilution). At regular time points the OD of an aliquot of broth was measured. In addition samples were taken from broth at the same times, serially log diluted (as described in section 5.2.3) and 4 x 10µl drops transferred onto quadrants of agar plates. Plates were read at 24 hours. Lag, log and stationary phases were determined.

5.2.5 Purification of MBL

MBL was purified from human, ethanol-fractionated plasma paste by the method of Kilpatrick (Kilpatrick, 2001) modified to include positive affinity removal of human immunoglobulin. Essentially, the sample was twice passed through mannan-agarose columns to isolate the protein, and the product was then passed through an anti-immunoglobulin column as a safeguard against immunoglobulin contamination. Briefly, 500 g of frozen ethanol-fractionated human plasma paste (donated by C. Dash, Blood Products Laboratory, Elstree, United Kingdom) was purified by ammonium sulfate precipitation to give 42% saturation. After dialysis the solution was applied to a mannan-agarose (Sigma, Poole, United Kingdom) column (5-ml packed volume; Pharmacia Biotech, Uppsala, Sweden) and the calcium-dependent proteins were eluted

with 0.01 M EDTA. The first EDTA eluate was recalcified to 0.05 M $CaCl_2$, reapplied to the same mannan-agarose column, and eluted with 0.1 M mannose. The concentration of MBL was determined by enzyme-linked immunosorbent assay, and sample purity was verified by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 3 to 10% polyacrylamide gradient gel and silver staining. MBL prepared in this manner is known to be noncovalently associated with MASP. This was kindly performed and donated by Dr Marina Johnston at ICH.

5.2.6 FITC conjugation of anti-MBL antibody

Conjugation was performed using a well established method in our laboratory. The storage buffer of a 1-mg/ml solution of monoclonal anti-MBL antibody (clone 131-1; State Serum Institute, Copenhagen, Denmark) was exchanged for conjugation buffer (see conjugation buffer in methods) using a centrifuge ultrafiltration device (Ultrafree MC; Millipore, Watford, United Kingdom) according to the manufacturer's instructions. To this a solution of fluorescein isothiocyanate (FITC; 1 mg/ml in conjugation buffer) was added (0.3 ml/ml of antibody solution), and the resulting mixture was incubated at room temperature for 3.5 h. The buffer was exchanged for phosphate-buffered saline (PBS) using an ultrafiltration membrane. Unconjugated FITC was removed by continuous washing with PBS until the buffer ran clear. The conjugated antibody was aliquoted and stored at 70°C until use. Antibody concentration (a) and efficiency of coupling (b) were determined by measuring absorbance at 280nm (protein) and 495nm (FITC) and using the following equations.

a) Antibody concentration = OD_{280nm}-(OD_{495nm} x 0.35) / 1.35mg/m

b) Efficiency of coupling = OD_{280nm} / OD_{495nm}

A coupling efficiency ratio of 0.5 – 1.0 indicates good coupling whilst a ratio of less than 0.5 indicates a poor conjugation. In this study the OD_{280nm} was 0.580 and OD_{495nm} was 0.686 giving a final antibody concentration of 0.25mg/ml and a coupling efficiency ratio of 0.85 demonstrating good conjugation.

Efficacy was tested using the MBL binding assay (see section 5.2.7) with an organism known to bind MBL. Fig 5.1 shows that approximately 90% of the organisms were positive for FITC with the new conjugated antibody compared to both an old antibody that had undergone multiple freeze/thaws and a control for background fluorescence (where the same FITC anti-MBL antibody, but no MBL, was used). This control also demonstrated that unbound FITC had been washed off during the conjugation process. FITC conjugation was therefore confirmed and this antibody was used in all subsequent experiments.

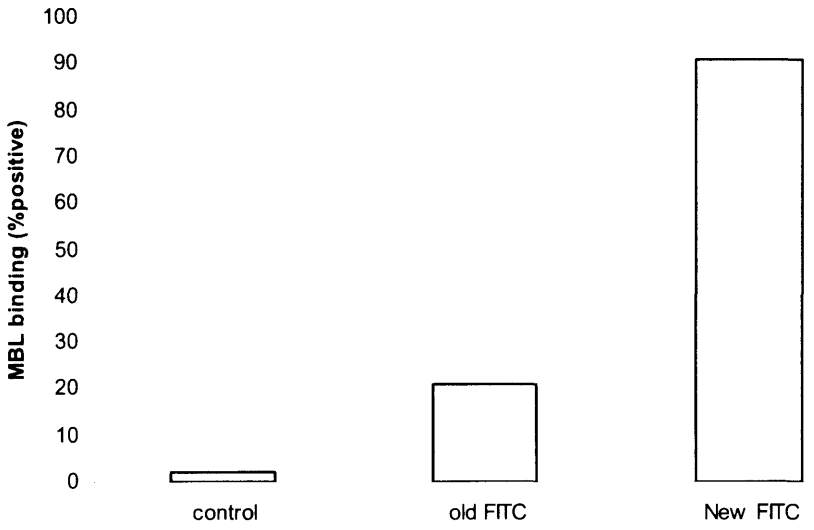


Fig 5.1 Comparison of "new" and "old" FITC conjugated anti-MBL antibody: Newly prepared FITC conjugated anti-MBL antibody was compared with a previous "old" antibody in an experiment binding MBL to an organism known to bind MBL. FITC conjugation was therefore confirmed.

5.2.7 Assay for MBL binding to microorganisms

The binding of MBL to all bacteria was determined by a flow cytometric procedure as described previously (Jack et al., 1998). A 50-µl aliquot of organism suspension at OD 1 (540nm) was centrifuged at 8300g for 5 minutes and the pellet was resuspended in HBBS++ containing 5µg/ml MBL or no MBL for a negative control. Suspensions were incubated at 37°C for 30 min and then respun at 8300g for 5 minutes. The supernatant was removed and the cell pellet was washed with 200µl HBBS++ before resuspension in 25µl of HBBS++ containing 10 µg/ml of FITC-conjugated anti-MBL antibody. This mixture was incubated at 37°C for 30 min before centrifugation at 8300g for 5 minutes. The supernatant was removed and the pellet washed with 200µl HBBS++ before resuspension in 150µl of PBS. The samples were fixed by the addition of 150µl of FACS fix for flow cytometry. Organisms were identified on the basis of size and granularity. Data were evaluated both as percentage of positive organisms and median fluorescent intensity (MFI).

5.2.8 Influence of MBL concentration on binding

To determine the concentration dependence of binding, 4 strains identified as high (3) and low (1) binders, were incubated with MBL at concentrations ranging from zero to 10 µg/ml. Concentrations were chosen to reflect both MBL deficiency, normal levels for a Caucasian population (approximately 1.6 µg/ml (Turner, 1996) and higher levels seen in an acute phase response (up to 10µg/ml).

5.2.9 MBL inhibition studies

To confirm that the binding of MBL was due to an interaction of the C-type lectin domain with bacterial sugars, competition experiments were performed using EDTA (a calcium chelater and known antagonist of MBL), a monosaccharide *N*-acetly-D-

glucosamine known to bind MBL and a sugar, galactose, known not to compete with MBL binding (Devyatyarova-Johnson M et al., 2000). In each case either 0.5 M EDTA, 0.25M *N*-acetly-D-glucosamine or 1 M galactose were added to the organism suspension for 10 minutes prior to the addition of MBL in a method described previously by Neth et al (Neth et al., 2000). The organisms used were BCC 18870 (GIV) and BCC 16656 (GIII).

B) The influence of MBL variant alleles on the acquisition of BCC in a clinical cohort of patients with cystic fibrosis

5.2.10 Population of CF patients with Bcc

MBL genotypes were known for the 13 patients from the RBH whose sputum isolates of Bcc were used in the MBL binding experiments (section 5.2.2 and 5.2.7) (Davies et al., 2000b). In addition I determined the MBL haplotypes on a further 38 patients colonized with Bcc from 3 other UK collaborating centers in Cambridge, Leeds and Belfast (samples kindly provided by Prof D Bilton, Dr S Conway and Prof S Elborn). Patient samples in the latter cases were anonymous, identified only by number, and therefore no further clinical data are available. Whole blood samples were provided and DNA extraction and MBL haplotypes were performed as described in chapter 2.

5.2.11 Evaluation of data and statistical analyses

MBL binding was determined by flow cytometry. Differences between MBL binding to different organisms were analysed by a Kruskal-Wallis test. In each case, comparisons were made with the background fluorescence in the absence of MBL (negative control) which was set at 2% by previous convention. Allele frequencies were estimated by gene counting. The goodness of fit between observed and expected allele frequency was statistically tested using a $\chi 2$ test.

5.3 Results

A) MBL binding to Bcc

5.3.1 Quantification of *Burkolderia cepacia* complex organisms in Hanks' balanced salt solution ($HBSS^{++}$) at an Optical Density (OD) of 1

Organisms were quantified using the method of Miles & Misra. A suspension of Bcc in $HBBS^{++}$ at an OD of 1 (540 nm) equated to approximately 1.5x 10^9 CFU/ml. This is of the same order of magnitude as other similarly sized gram negative organisms (Ison et al., 1995).

5.3.2 Bacterial Growth Properties

When grown in broth the time course was as seen in Fig 5.2A. A lag (approx 0-4 hours), log (approx 4-12 hours) and stationary phase (approx 12-20 hours) can be determined. The no of CFU grown when samples were taken from the broth and plated onto agar is shown in Fig 5.2B. Again the organisms seem to be in stationary phase around 16-18 hours. This confirms a similar growth time course of Bcc to other bacteria previously used in MBL binding experiments.

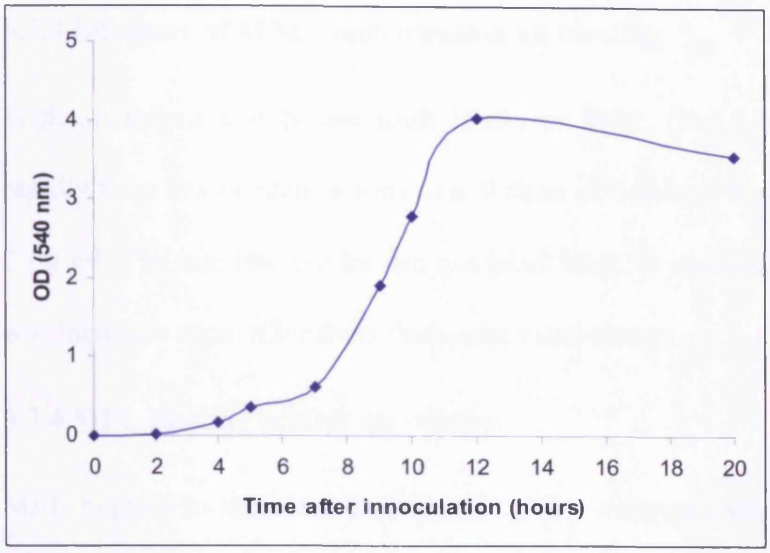


Fig 5.2 A

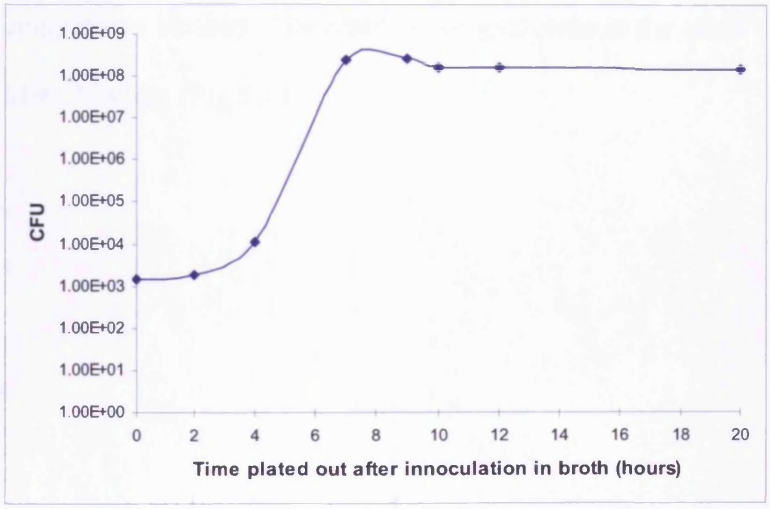


Fig 5.2 B

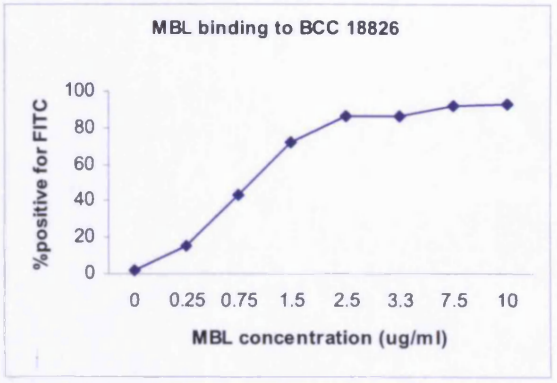
Fig 5.2. A) Growth of BCC 18870 (G IV) in brain heart broth. Turbidity of broth was measured by measuring the optical density (OD) at 540 nm. **B) No of CFU of BCC 18870.** Samples were taken from broth at times shown, serially log diluted and 4 x 10µl drops transferred onto quadrants of agar plates using the methods of Miles and Misra. Plates were read at 24 hours.

5.3.3 Influence of MBL concentration on binding

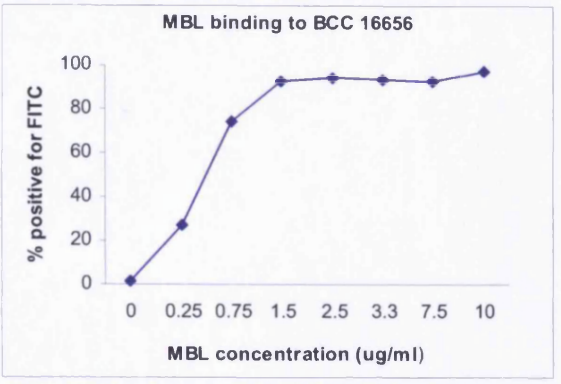
With organisms that bound high levels of MBL (Fig 5.3 A,B,C) binding increased rapidly from low concentrations to a plateau of maximal binding between 2.5 μg/ml and 5 μg/ml. The one low binder did not bind MBL at any concentration. 5μg/ml of MBL was therefore chosen for the subsequent experiments.

5.3.4 MBL binding inhibition studies:

MBL binding to both Bcc 18870 and 16656 was markedly reduced in the presence of EDTA, demonstrating the divalent-cation (calcium) dependent nature of the observed interactions. In addition *N*-acetyl-D-glucosamine inhibited binding of MBL due to competitive binding. The addition of galactose in the same experiments had no effect on MBL binding (Fig 5.4).



A



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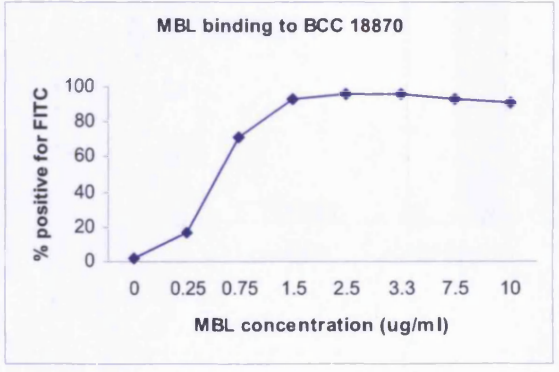
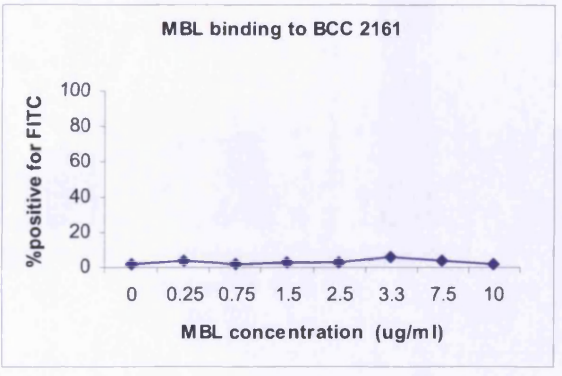


C



D

Fig 5.3. The binding of MBL to *Burkholderia cepacia* organisms is dependent on MBL concentration. Bcc organisms **A)** 18826, **B)** 16656 (both G III), **C)** 18870 (G IV) and **D)** 2161 (G I) are shown. **A, B & C** bind strongly at physiological concentrations of MBL, with binding increasing rapidly from low concentrations to a plateau of maximal binding at about 2.5µg/ml. Bcc 2161 **(D)** did not bind MBL at any concentration.

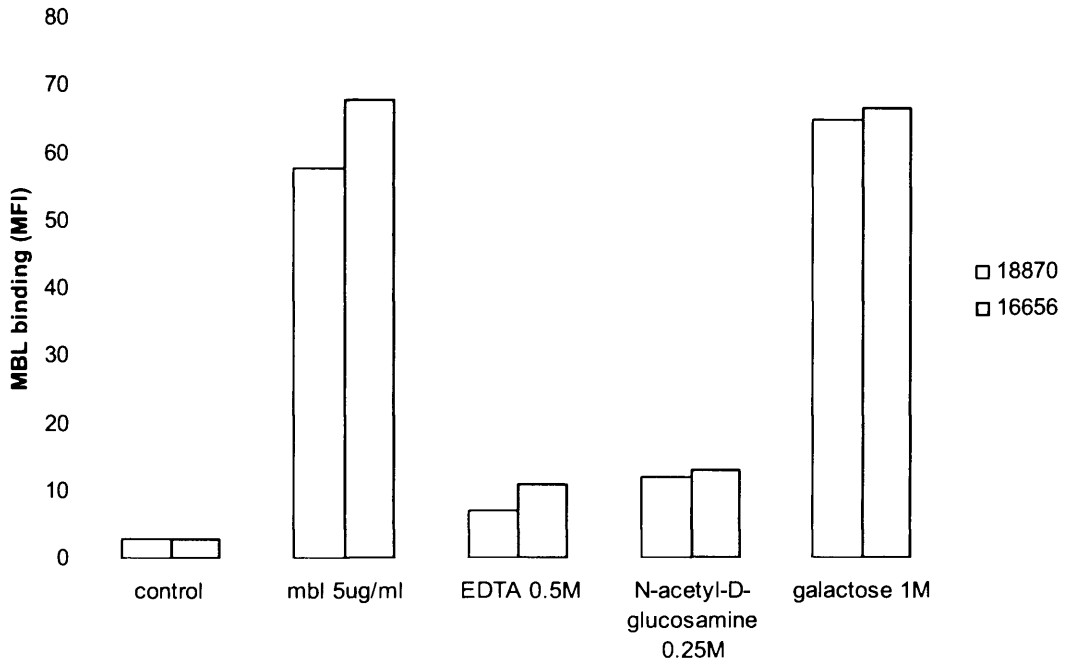


Fig 5.4. Specificity of MBL binding to two Bcc strains 18870 & 16656.

Binding of MBL to Bcc organisms 18870 and 16656 is inhibited by preincubation with EDTA & *N*-acetylglucosamine but not by galactose.

5.3.5 MBL binding to Bcc organisms isolated from CF patients

MBL binding was demonstrated to the majority of the clinical isolates (11/13) with two of the organisms showing minimal binding (no's 11 and 12) (Fig 5.5). Both of the ET 12 strains (no's 9 and 10) bound MBL.

The source of the organisms was from a population in which there was an overrepresentation of MBL structural alleles (8/13, 62%) (Davies et al., 2000b) and low MBL serum levels (9/12 (75%) <1000ng/nl). However the patients own serum level did not affect whether they were infected with an organism that bound or did not bind MBL; Pearson's coefficient of linear correlation r = 0.2, p=0.5 (Table 5.2 & Fig 5.6).

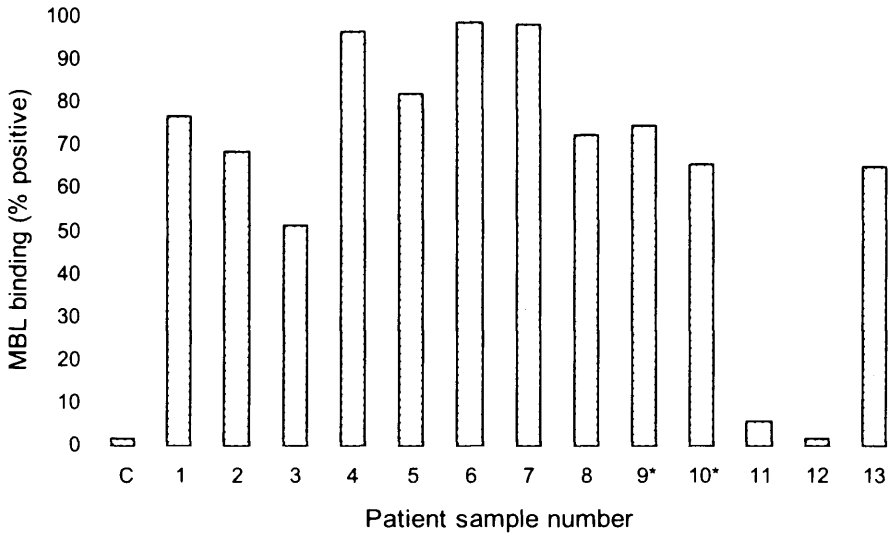


Fig 5.5 MBL binding to 13 Bcc strains isolated from CF patients. Data are expressed as percentage of organisms positive for FITC (samples incubated with MBL and FITC-anti MBL antibody). Each organism was compared to its own control (incubated with FITC-anti MBL antibody alone). Each control was set at 2% so only one (C) is shown for clarity. Mean MBL binding 66%; range 2-98%). * are ET12 strains.

Patient number	MBL Genotype	MBL Serum level	MBL binding (% positive) to patient's Bcc isolate	ET 12 strain Y/N
1	XAO	<100	77	N
2	XAO	450	69	N
3	OO	<50	52	N
4	XAO	<100	97	N
5	YAYA	1877	83	N
6	YAO	<100	99	N
7	YAYA	5555	99	N
8	YAO	180	73	N
9	YAXA	No serum	75	Y
10	YAYA	2872	66	Y
11	YAXA	738	6	N
12	YAO	702	2	N
13	YAO	244	66	N

Table 5.2 Relationship of patient MBL genotype, MBL serum level, presence or not of Bcc ET12 strain and MBL (5 μg/ml) binding to patients own Bcc isolate.

In this series of 13 cases the binding of purified MBL (5 μg/ml) to the patients own clinical isolate of Bcc was determined by flow cytometry.

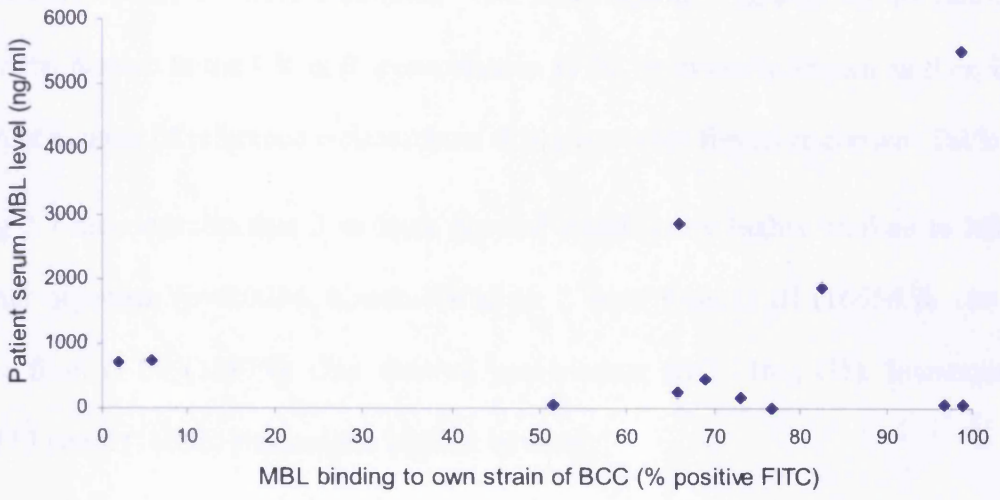


Fig 5.6 Relationship between MBL binding to patients own strain of Bcc and patients serum MBL level. 12/13 patients had serum available. 9/12 had serum levels < 1000ng/ml. An MBL concentration of 5μg/ml was used in the binding experiments. There was no correlation between patients own serum level and whether they were infected by an organism that bound or did not bind MBL (Pearson's r = 0.2, p=0.5).

5.3.6 MBL binding to different BCC genomovars

To investigate if a specific Bcc genomovar influenced MBL binding, 12 strains from genomovars (GI-V) were examined. The most common genomovar to cause severe clinical disease in the UK is *B. cenocepacia,* G III, (previously known as *B.cepacia).* A larger number of reference isolates from this group were therefore chosen (Table 5.3).

Fig 5.7 demonstrates that 3 isolates showed significantly higher binding to MBL than other organism (p=0.0034, Kruskal-Wallis). 2 were from G III (16656 & 18826) and one from G IV (18870). One showed low binding (BC 2161; G1). Interestingly the ET12 strain (16656) showed the highest binding.

Genomovar	Species name	Identification Number & Source of Isolate
I	*B. cepacia*	1222 (onion), 2161 (soil)
II	*B. multivorans*	18822 (CF), 13010 (CF)
III	*B. cenocepacia*	18826 (CF), 16656 (CF ET12 strain), 16654 (CF), 18832 (UTI)
IV	*B. stabilis*	14294 (CF), 18870 (CF)
V	*B. vietnamiensis*	10929 (soil), 18836 (CGD)

Table 5.3 Genomovars and strains of Burkholderia cepacia used in the MBL binding experiments.

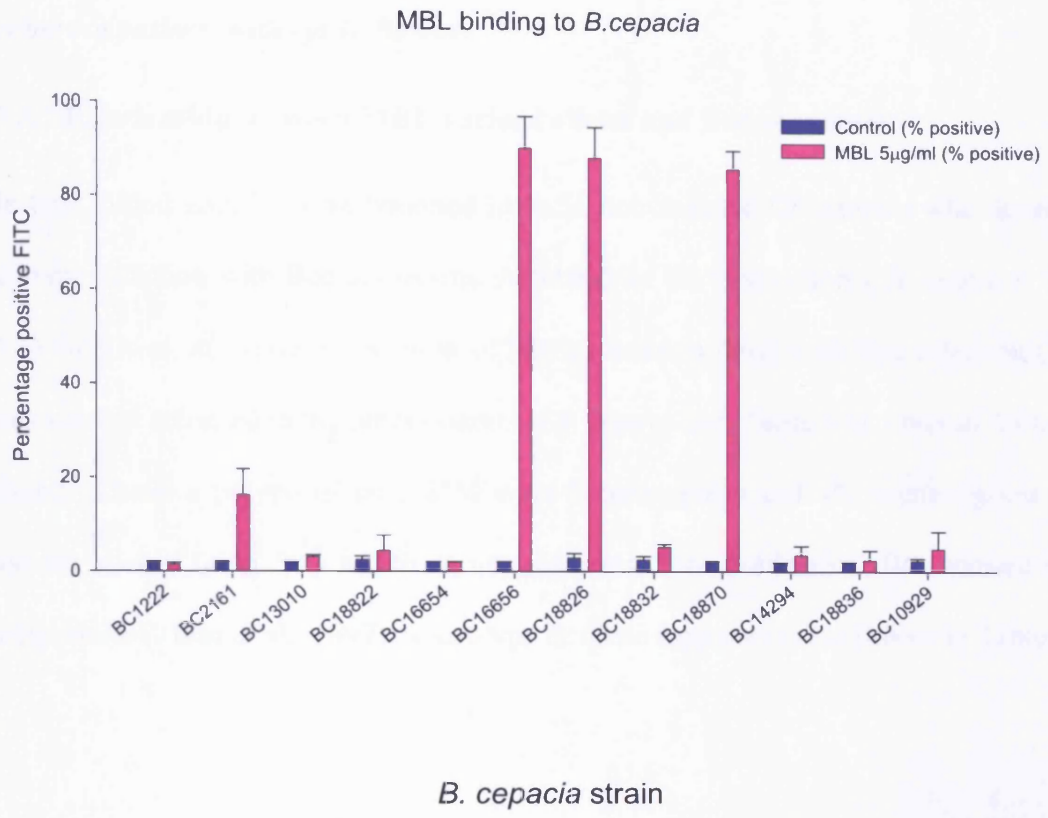


Fig 5.7 MBL binding to known strains of different genomovars of Bcc.

Data are expressed as percentage of organisms positive for FITC (samples incubated with MBL and FITC-anti MBL antibody). Each organism was compared to its own control (incubated with FITC-anti MBL antibody alone). Each control was set at 2%. Each column represents the mean of at least three experiments, error bars show one standard deviation. Binding to different organisms was significantly different; p=0.0034 Kruskal-Wallis test.

B) The influence of MBL variant alleles on the acquisition of BCC in a clinical cohort of patients with cystic fibrosis

5.3.7 Relationship between MBL variant alleles and Bcc acquisition

In total, blood samples were obtained from 51 non selected CF patients who developed chronic infection with Bcc organisms. Although in the first cohort (CF centre 1, Table 5.4) there was an overrepresentation of MBL alleles in those with Bcc infection (62%) this was not reflected in the other cohorts (CF centres 2-4, Table 5.4). Overall 45% were found to have a polymorphism, 41% were heterozygotes and 4% homozygotes for a variant allele (Table 5.4) which is not different from published MBL variant allele frequencies (Mead et al., 1997). Genotype & allele frequencies are shown in Table 5.5.

CF Centre	**No of patient samples**	**No (%) MBL heterozygotes (A/O)**	**No (%) MBL Homozygotes (O/O)**	**Total no (%)MBL polymorphisms (A/O + O/O)**
1	13	7 (54%)	1 (8%)	8 (**62%**)
2	21	8 (38%)	1 (5%)	9 (43%)
3	11	4 (36%)	0 (0%)	4 (36%)
4	6	2 (33%)	0 (0%)	2 (33%)
Total	**51**	21 (41%)	2 (4%)	23 (**45**%)

Table 5.4 Analysis of MBL variant alleles in 51 patients with cystic fibrosis and Bcc infection. These patients were recruited from 4 UK regional CF centers. Patients from centers 2-4 were completely anonymous and therefore no further data are available.

Genotype	Frequency in Bcc patient population (%)	Frequency in UK "control" population (%)*	Frequency in UK CF population (%) #
A/A	55	59.6	61.1
A/O	41	35.8	35.2
O/O	4.0	4.6	3.8
A/O + O/O	45	40.4	39

Table 5.5 A

Allele	Frequency in Bcc patient population	Frequency in UK "control" population *
Wild type (A)	0.755	0.775
Codon 52 mutation (D)	0.040	0.066
Codon 54 mutation (B)	0.166	0.144
Codon 57 mutation (C)	0.040	0.015

Table 5.5 B

Table 5.5 shows genotype (A) and allele (B) frequencies for the 51 patients with *Burkholderia cepacia* complex infection. These frequencies are not significantly different from those published in a UK well "control" population and in our UK CF population (p=0.94 Chi-square). *(Mead et al., 1997), #(Davies et al., 2004) and chapter 3.

5.4 Discussion

Infection with Bcc usually occurs in patients with cystic fibrosis who are already colonized with *Pseudomonas aeruginosa* and have background lung disease. Why only a small proportion of patients acquire this organism and why some have a more severe clinical course is unknown. Both bacterial factors and the host response are thought to play a role. Certainly the propensity for the acquisition of *B. cenocepacia* and *B.multivorans* by CF patients would suggest some differences in virulence factors between genomovars that are of particular importance in the CF lung. The way that Bcc organisms are handled by parts of the innate immune system, such as MBL, were thought to be important following the effect seen of MBL variant alleles on acquisition of Bcc in CF patients in preliminary small cohorts (Garred et al., 1999b;Davies et al., 2000b).

In two small previous studies by Garred (n=10) (Garred et al., 1999b) and Davies (n=13) (Davies et al., 2000b) an association between Bcc infection and MBL genotype was observed. In the study presented here 51 patients with cepacia (including the 13 patients from the RBH (Davies et al., 2000b)) were genotyped for MBL polymorphisms. There was no significant difference in the overall number of variant alleles in this cohort compared to published data from a prospectively recruited UK population of children being followed up for the ALSPAC study (Mead et al., 1997) and the large UK CF population presented in chapter 3 (Davies et al., 2004) (p=0.94, chi-square). The observed genotype frequencies of the Bcc group as a whole were not significantly different from those expected as calculated by the Hardy Weinberg equilibrium equation.

From the work presented in this chapter, MBL deficiency does not seem to predispose CF patients to Bcc infection. This is consistent with the finding that despite worse lung

disease, MBL deficient CF patients are also not at increased risk of acquisition of *P. aeruginosa*, as shown in Chapter 3 and also by Garred et al (Garred et al., 1999b;Davies et al., 2004). Taken together, these findings may suggest that complement mediated clearance of bacteria is not sufficient to prevent infection with these two organisms. However MBL could be operating in a number of ways. Although not sufficient to prevent colonization, MBL could still limit infection or reduce bacteria within the lungs. Indeed a recent report highlights a case of chronic Bcc infection in an MBL deficient *non* CF individual (Whitehouse et al., 2005). In the cohort presented here insufficient clinical or microbiological data were available to analyse this aspect further. However as the capacity of Bcc to bind MBL is likely to be a prerequisite for any MBL mediated antimicrobial activity, its capacity to bind to the organism was explored.

11/13 (85%) of the clinical isolates of Bcc showed significant binding to MBL. Bcc have been subdivided into genomovars on the basis of physical and genotypic characteristics. To investigate if these bacterial properties were important in determining MBL binding, 12 Bcc reference strains representing 5 different genomovars were examined. No clear pattern emerged of MBL binding to the specific genomovars found commonly in CF. Only 2/4 G III, and 1/2 G IV organisms bound MBL but interestingly binding was highest to the genomovar III ET12 strain. Binding of organisms to MBL showed similar MBL concentration dependence and inhibition as seen with another gram negative organism, *Salmonella* serovar Typhimurium (Devyatyarova-Johnson M et al., 2000).

Bcc organisms are known to have multiple virulence factors including bacterial haemolysins (Hutchison et al., 1998), proteases (Kooi et al., 1994) and siderophores (McKenney and Allison, 1995). Lipopolysaccharide (LPS) from Bcc organisms is particularly pro-inflammatory and has been shown to elicit a nine fold higher release of

cytokines from leukocytes compared to that of *P. aeruginosa* (Shaw et al., 1995). Genomovar specific pro-inflammatory cytokine (TNF-α & IL-1β) production has been looked at *in vitro* and shown varying biological activity within and between genomovars that was probably related more to components of LPS than to genomovar. Interestingly significantly more GIII isolates (including BCC LMG 16656 & 18826) elicited above average TNF-α production (De Soyza et al., 2004). MBL binding to organisms is also known to be influenced by LPS structure (Devyatyarova-Johnson M et al., 2000;Jack et al., 1995). As genomovars are not defined by LPS, it is perhaps not surprising that MBL binding does not occur in a genomovar specific pattern.

In spite of the mixed binding pattern of the reference strains, 11 of the 13 clinical strains did bind MBL. Although it appears unlikely that MBL is affecting Bcc acquisition it is possible that it can modulate the host's inflammatory response to this and other bacteria. The next chapter investigates how MBL can affect the development of systemic inflammation in response to an acute infectious or non-infectious insult.

CHAPTER 6

The role of MBL in Inflammation

6.1 Introduction

Multiple organ failure resulting from systemic inflammation remains the predominant cause of morbidity and mortality on intensive care, irrespective of the initial illness or insult precipitating admission (Vincent, 1996;Marshall, 2001). Individuals differ considerably in their response to an infectious or traumatic insult which could be explained by polymorphisms of the genes encoding proteins involved in this response. Seminal work by Sorensen et al (Sorensen et al., 1988) showed that death from infection of a biological parent under the age of 50 years, conferred a 5.8 fold increased risk of dying on the offspring. In this study the genetic contribution to this risk was more than seen in cardiovascular disease or cancer. Exactly which genes are involved are as yet unknown but are likely to include those involved in innate immunity, coagulation and inflammatory pathways (Sutherland and Russell, 2005). In children, mortality is low and therefore markers of morbidity such as severity of illness, multi-organ failure and the systemic response to an insult, the systemic inflammatory response syndrome (SIRS), must be examined.

The work described in this thesis so far has demonstrated that MBL can bind to organisms (chapter 5), can be found in extra-vascular sites (chapter 4) and can modulate disease severity (chapter 3). It was therefore hypothesised that MBL is also involved in the modulation of inflammation. This hypothesis largely arose from work by Jack et al which showed, in an ex vivo whole blood model, that the addition of MBL to the blood of MBL-deficient donors influenced the production of monocyte-derived inflammatory

cytokines after bacterial stimulation. Specifically, high concentrations of MBL (>6000ng/ml), such as seen in an acute phase response, decreased the production of interleukin-6 (IL-6), interleukin -1β (IL-1β) and tumour necrosis factor-α (TNF-α) by monocytes in response to meningococci, whereas lower concentrations (<1000-2000ng/ml), as seen in MBL homo or heterozygotes, enhanced the production of IL-6 and IL-1β (Jack et al., 2001b) (See section 1.3.4).

In this chapter, the role that MBL may play in a clinical setting in which inflammation occurs in both the presence and absence of infection was examined. To do this a study was designed to look at the association of MBL haplotypes and serum levels with the development of both sepsis and/or SIRS in admissions to a PICU (part A). In addition experiments were performed to explore ex-vivo cytokine responses to bacteria in the presence or absence of MBL (part B).

6.2 Methods

A) The effect of MBL on the systemic inflammatory response syndrome (SIRS) and sepsis in paediatric intensive care patients

6.2.1 Patient Selection

Consecutive admissions to the tertiary multi-disciplinary PICU at Great Ormond Street Hospital were recruited to this study over a 10 month period in 2002. Inclusion criteria were: age between birth and 17 years; presence of at least one organ system failure for > 12 hours (or death within the first 12 hours). Organ failure was defined as follows; respiratory failure: the need for assisted ventilation (mask CPAP or intubation), cardiovascular failure: the need for inotropes following > 40ml/kg/day of resuscitation fluid, renal failure: the need for dialysis or haemofiltration, central nervous system failure: a Glasgow coma scale (GCS) of < than 8/15. The following exclusions were applied: presence of multiple congenital abnormalities; known congenital immunodeficiency; known central neurological or neuromuscular disease (all considered to represent major risk factors for ICU admission resulting from infection); persistent pulmonary hypertension of the newborn, weight < 2.2 kg, informed consent not available; suspected non-accidental injury; repeat PICU admission during the study period; lack of intravenous or intra-arterial access and anticipated short stay of <24 hours (eg high dependency patients or those ventilated briefly and routinely post operatively) on the PICU.

On enrolment, cases were assigned to one of two groups according to the principal reason for ICU admission as documented for audit purposes by ICU physicians not involved in the study. The groups were: 1) infection (presumed or proven), 2) non-infection (trauma, post operative or 'other'). The primary outcome measure within each

group was the development or not of SIRS within 48 hours of admission by age-adjusted criteria.

Infection was defined as 'proven' if a causative organism was isolated and 'presumed' in those with a history and examination consistent with an infection e.g. fever, cough and coryza combined with chest x-ray changes consistent with pneumonia. Diagnoses of SIRS, sepsis and septic shock were made according to 1992 ACCP/SCCM guidelines modified for age (Bone et al., 1992;Hayden et al., 1993). In brief, SIRS was determined by the presence of ≥2 of the following: central temperature >38.0°C or < 36.0°C, white cell count >12x10^9/l or <4x10^9/l and a heart rate (beats per minute, bpm) outside age specific ranges (newborn to 3 months: 95-145 bpm, 3-12 months 110-175 bpm, 1-<3 years 105-170 bpm, ≥3-<7 years, 80-140 bpm, ≥7-<10 years 70-120 bpm, ≥10 years 60-100 bpm). Respiratory rate was not included as a diagnostic criterion because of the very high proportion of cases receiving mechanical ventilation. Cases meeting these criteria for SIRS with 'proven' or 'presumed' infection were classified as 'Sepsis'. Septic shock was diagnosed in cases of sepsis who were hypotensive, defined against age specific values for mean blood pressure (newborn to 3 months: 40-60 mmHg, >3-<6 months 45-75 mmHg, ≥6 - <12 months 50-90 mmHg, ≥1-<3 years 50-100 mmHg, ≥3-<7 years, 50-100 mmHg, ≥7-<10 years 60-90 mmHg, ≥10 years 65-95 mmHg) after fluid resuscitation, requiring treatment with inotropic and/or vasopressor therapy (≥ dopamine 5μg/kg/min, any dose of epinephrine, norepinephrine or vasopressin).

An electronic patient charting system (Care Vue, Hewlett Packard) was in use on PICU in which clinical and laboratory parameters were recorded on at least an hourly basis. For study patients these values were reviewed regularly by a PICU consultant (Dr Mark Peters) or senior clinical fellow (Dr Peter Wilson) and maximum and minimum ventilator and physiological parameters for each 24-hour period were recorded.

Microbiological, biochemical and haematological information was recorded from the laboratories at GOSH and the referring hospital if available. A severity of illness score, the Paediatric Index of Mortality (PIM) which estimates the risk of death from the severity of physiological disturbance, was assessed on first contact with the PICU team (Shann et al., 1997). Another severity of illness score; the sequential organ failure assessment (SOFA) score, was also calculated (section 6.2.8). All clinical information obtained was organised into a designated, password secured, access database and subsequently transferred into the statistical programmes SPSS (Version 12.00) and/or PRISM (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA) prior to analysis.

6.2.2 Consent & recruitment

I or one of my colleagues, a PICU consultant (Dr Mark Peters) or a senior clinical fellow (Dr Peter Wilson) reviewed all paediatric and neonatal intensive care admissions almost every day that the study was running to identify possible patients for inclusion. All of the initial patient recruitment and consent was done by myself or one of these doctors, however I was subsequently unaware of the patients final diagnosis or whether or not they developed SIRS.

6.2.3 Ethical Approval

The study was approved in advance by the Ethics Committee of Great Ormond Street Hospital. All parents or carers gave informed consent for participation.

6.2.4 Blood sample processing

2-3 mls of whole blood was collected, at a time of a clinical sample being taken and divided into an EDTA and serum collection tube. Samples were taken within 48 hours, (usually within 8 hours) of admission. Whole blood in EDTA was stored at -20°C until

DNA extraction was performed. Serum samples were spun, separated and the serum stored in aliquots at -80°C until analyzed.

6.2.5 Mannose binding lectin genotyping and serum analysis

Preparation and amplification of genomic DNA, and *MBL-2* haplotyping were performed as previously described (Section 2.3.3). MBL serum levels were measured by a symmetrical sandwich ELISA using commercial kits (Antibody Shop, Copenhagen, Denmark) according to the manufacturer's instructions. Serial MBL serum levels were performed using the "in house" ELISA (Section 2.3.1).

The investigator performing the MBL genotype and serum levels (KF) was blinded to the diagnosis of SIRS/non SIRS. Likewise the clinician (PW) who acquired the clinical data did so before the results were analysed and was therefore also blinded to the outcome.

6.2.6 CRP measurement

This procedure was performed by staff in the Biochemistry Department at GOSH by an enzymatic sandwich immunoassay (Vitros CRP).

6.2.7 Serial MBL serum measurements

A small number of children were recruited for serial measurements of serum MBL. Blood samples were obtained, daily where possible, whenever a routine clinical sample was taken to avoid unnecessary venupuncture or access of central lines.

6.2.8 Severity of illness scores

In children mortality is low and therefore markers of morbidity such as severity of illness are required for studies such as these. To help determine a patients' outcome at the beginning of, or during, an illness a number of scoring systems have been devised to stratify patients into high or low risk groups. These include the paediatric index of mortality (PIM), from which a risk of mortality can be calculated and the Sequential Organ Failure Assessment (SOFA) score which is calculated daily to determine progression or resolution of multiorgan failure. Both involve complex mathematical equations to translate physiological data into a score. Table 6.1 demonstrates the physiological parameters required to calculate these scores.

	Parameters used to calculate the risk of mortality or to describe the severity of illness. Yes=√	PIM	SOFA
	Elective admission	√	
	Underlying condition	√	
Central nervous system	Response of pupils to bright light	√	
	Glasgow coma scale		√
Respiratory	Mechanical ventilation	√	√
	FiO2 (%) / PaO2 (mmHg)	√	√
	PaCO2		
Cardiovascular	Heart Rate		
	Systolic Blood Pressure (mmHg)	√	√
	Base excess (mmHg) (arterial or capillary blood)	√	
	Use of inotropes		√
Hematological	White blood cell count		
	Platelet count		√
	INR or prothrombin time		
Renal	Creatinine		√
Liver	SGOT		
	Bilirubin		√

Table 6.1 Parameters used to calculate the PIM and SOFA scores. These scores are based on the severity of physiological disturbance. The PIM score also uses information on whether or not the patient is an elective admission and/or has an underlying condition. All describe morbidity. PIM = paediatric index of mortality, SOFA = sequential organ failure assessment.

B) The effect of MBL on the production of cytokines by monocytes

6.2.9 Meningococcal strains

Two strains of serogroup B meningococci were used in this study, B1940 and H44/76 (B:15:P1.7,16:) (Holten, 1979). Both are encapsulated and express the L3 immunotype. In addition, two galE mutants each derived from the respective parent strains were used. The galE strains were made by homologous recombination using a chloramphenicol resistance cassette inserted in the galE gene, which encodes the enzyme UDP-4 galactose epimerase (Hammerschmidt et al., 1994). The resulting mutants are encapsulated but express a truncated LOS exposing a terminal glucose that lacks the lacto-neotetraose moiety of the alpha chain and cannot be sialylated. The mutants are piliated and express outer membrane proteins opa and opc (Hammerschmidt 1994). Prior to use, bacteria were heat inactivated at 56 ^{o}C for 30 minutes. All of these killed organisms were kindly provided by Dr Garth Dixon, Clinical Scientist, ICH.

6.2.10 Mannose binding lectin

MBL was purified from human plasma (as described in chapter 5.2.5) and used at the concentrations stated.

6.2.11 To determine MBL binding to wild type meningococci and the gal E isogenic mutant

To confirm that MBL did indeed bind these meningococcal organisms, as demonstrated in many previous reports (Jack et al., 1995), binding experiments were performed as described in chapter 5.2.7. In the experiment performed here 50µl of 1 x 10^{9}/l of organism suspension (approximately 5 x 10^{7} organisms) was incubated with 5µg/ml of MBL.

6.2.12 Whole blood collection

The required amount of whole blood was collected from laboratory volunteers (usually approx 20 mls) via a single venupuncture. Samples were placed immediately into sterile 5ml bijous containing preservative free heparin (final concentration 10 U/ml). To avoid *ex vivo* activation, all blood samples were processed immediately. MBL deficient donors were used for the relevant experiments.

6.2.13 Assay for intracellular monocyte cytokine production

Whole blood culture

Whole blood from persons known to have little or no serum MBL was placed in a vial containing anti-coagulant. This was initially heparin sodium (final concentration 10iu/ml) and in subsequent experiments sodium citrate (final concentration 0.76% wt/vol). Brefeldin A (Sigma, Steinheim, Germany) was added to a final concentration of 10μg/mL. Brefeldin A interferes with the export of proteins from the Golgi apparatus and causes the intracellular retention of proteins that would normally be secreted (Misumi et al., 1986). 500μL of blood was aliquoted into polypropylene tubes, and purified MBL (final concentration 0-8μg/mL) was added. The blood was then diluted with an equal volume of RPMI 1640 medium containing unlabelled meningococci to a final concentration of 5×10^6 organisms/mL or only medium (unstimulated control). Blood was incubated for 3 hours at 37°C with 5% CO_2.

Monocyte Surface Staining

Aliquots of the whole blood mixture (200μL) were taken after 3 h and incubated with 5μL of FITC conjugated monoclonal anti-CD14 antibody (IgG2a, Serotec) (final concentration of 5μg/ml) or 5μL of an isotype control, for 30 min at room temperature in the dark. The erythrocytes were subsequently lysed by the addition of 2 mLs of FACS lysing buffer (Becton Dickenson, San Jose, USA) for 10 min. The remaining cells were

pelleted by centrifuging at 350g for 5 minutes, the supernatant was decanted and cells washed in 2 mLs of "wash buffer" of phosphate buffered solution (PBS) containing 0.5% Bovine Serum Albumin (BSA, Sigma, Steinheim, Germany) and 0.02% sodium azide (BDH, Poole, England) and repelleted by centrifugation at 350g for 5 minutes.

Cell membrane permeabilisation and intracellular cytokine staining

After surface staining and washing the cells were fixed by pellet resuspension in 200μl of 4% wt/vol paraformaldehyde in PBS for 10 minutes at room temperature. The samples were centrifuged, and the cell pellet was rewashed with "wash buffer" and recentrifugation. Cells were resuspended in 50μL of Leucoperm (Serotec Ltd, Oxford, England) followed by repeat washing. This permeabilisation step was critical to allow the cytokine specific monoclonal antibodies to penetrate the cell membrane, cytosol and endoplasmic reticulum.

The production of intracellular cytokines was assayed as per manufacturer's instructions using phycoerythrin (PE) conjugated mouse anti-human monoclonal antibodies to IL-6, TNF-α (Becton Dickinson), or IL-1β (R&D Systems). In brief 5μL of anti-cytokine antibody or 0.5μL of the isotype control were added to the cell suspension, and the mixture was incubated at room temperature for 30 min in the dark. Samples were then washed twice with wash buffer before resuspension in 400μL of Cellfix (BD) for flow cytometry. For each antibody an isotype matched control was used to control for non-specific binding. To conserve fluorescence, samples were protected from the light and stored at 4°C until analysed. Jack et al had previously shown by flow cytometry that the concentration of citrate used in this whole-blood model did not influence the binding of MBL (Jack et al., 1998).

6.2.14 Cytokine ELISAs

During the same whole blood experiments extra aliquots were set up in an identical manner and supernatant was removed after 3 hours of stimulation and stored for later cytokine anlaysis. ELISAs were performed for IL1β, IL-6 and TNF-α, according to manufacturer's instructions.

6.2.15 Flow cytometry

Flow cytometry allowed identification of monocytes from whole blood samples based on the cell size and granularity, in addition to the presence of fluorescence labeled monoclonal antibody to CD14. Prior to experiments being undertaken, the positions of cells were confirmed using the whole blood method but staining the cells with monoclonal antibodies to CD14 (monocyte marker), CD11b (neutrophil marker) and CD3 (lymphocyte marker) (data not shown).

6.2.16 FACS analysis

Flow cytometric analysis was performed on a FACSCalibur and analysed using CellQuest software (Becton Dickenson UK Ltd, Oxford, UK). Laser excitation was at 488nm and data were collected on FITC fluorescence at 525nm and PE fluorescence at 575nm. Forward and side scatter measurements were made with gain settings in linear mode and the monocyte population could be easily identified. Accuracy of the gate was confirmed by staining with a FITC conjugated antibody against the surface antigen CD14. Only cells that were both of the correct size and granularity, and stained with $CD14^{+}$ monoclonal antibody were collected for analysis. 5000 gated events were collected for analysis.

6.2.17 Confirmation of cytokine detection after whole blood stimulation with LPS

To ensure that the "system" worked and for acquisition of the technique, the experiment was initially performed using neisserial lipopolysaccharide at 1 and 50 ng/ml as the stimulus (kind gift from Jenner vaccine laboratory, Compton, Berkshire; extracted from serogroup B strain 44/76 by phenol chloroform extraction followed by purification). Antibodies to CD14, TNFα, IL-6 and IL-1β were used as above. Isotype controls for each cytokine were included. Unstimulated and stimulated $CD14^+$ cells were analysed for each cytokine. The amount of cytokine produced from the stimulated cells were compared to both the unstimulated cells and to background fluorescence from non specific binding ie the isotype control.

6.2.18 Statistical analysis

Data were analysed using SPSS v 12.00 and/or PRISM (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA). Observed and expected genotype frequencies were analysed by the Chi square ($\chi 2$) test. Differences between MBL serum levels were analyzed with the Mann-Whitney U test (for 2 groups) and the Kruskal-Wallis test (3 or more groups). Comparisons of the proportions of different MBL genotypes between clinical groups were performed using Chi square test. To determine the independence of any association detected, logistic regression techniques were used to determine the impact of the covariates MBL genotype, age, sex and ethnicity on the development of SIRS (or sepsis). Similarly MBL serum levels (rather than genotype) were analysed. Relationship of PIM and SOFA score to SIRS, sepsis and mortality was also assessed by logistic regression techniques.

6.3 Results

A) The effect of MBL on the systemic inflammatory response syndrome (SIRS) and sepsis in paediatric intensive care

6.3.1 Patient Demographics

After informed consent 147 cases were available for study. Three had no blood available and 2 were actually repeat admissions for which only the first admission was included for analysis. 142 patients were therefore available for full analysis of MBL genotype of whom 139 also had serum available for MBL serum levels & CRP. Ninety (63%) of the cases were male and the median age was 25.5 months (2.1 years).

Patient diagnoses were as follows: 1) Infection n=69 (48.6%), of whom 19 (27.5%) had localised infection only, 24 (34.8%) had sepsis and 26 (37.7%) had septic shock; 2) Non-infectious etiology n=73 (51.4%) including head injury trauma n=33; post surgical n=34; other n=6 (2 necrotising enterocolitis; 1 acute exacerbation SLE; 1 acute demyelinating encephalomyelitis(ADEM); 1 drowning and 1 with drug related Stevens Johnsons' syndrome). These were sub divided into those with, n=83 (58.5%) and without n=59 (41.5%) SIRS, irrespective of the aetiology. 134 of the 142 children were ventilated for >12 hours and 2 others were ventilated but died within 12 hours of admission. The remaining 6 were not ventilated but had at least one organ failure. The characteristics of the patients studied in relation to MBL exon 1 polymorphisms are shown in Table 6.2

	All Cases n=142	Wild type AA n=91	Hetero/homozygous variant A/O, O/O n=51	*P* value
Male, no. (%)	90 (63%)	59 (65%)	31 (61%)	0.63 $\chi 2$
Age, median (IQR), **(months)**	25.5 (7.5-121)	32 (10.5-127)	19 (7-103)	0.2 MW
Caucasian: **non Caucasian**	99:43	60:31	39:12	0.19 $\chi 2$

Table 6.2 Characteristics of patients studied and *MBL-2* exon 1 genotype.

There were no significant differences in patient age, sex or ethnicity between those with wild type (A/A) and variant (A/O, O/O) MBL alleles.

6.3.2 Mannose binding lectin genotypes

MBL genotype and haplotypes were successfully performed on all 142 patients. In addition haplotypes were performed on the 2 repeat admissions, excluded from analysis, to allow 2 "internal controls". In both of these cases the haplotype was the same, as expected, on both occasions.

Overall 91/142 (64.1%) had no structural *MBL-2* gene mutation (ie. wild-type and designated conventionally as A/A). 49 (34.5 %) of subjects were heterozygous for structural mutations (designated A/O) and 2 (1.4%) were either homozygous for one mutation or were compound heterozygotes (both designated O/O). Overall allele frequencies were as follows; wildtype (A) 0.813, codon 54 (variant B) 0.085, codon 57 (variant C) 0.042 and 52 (variant D) 0.060. This distribution was in Hardy-Weinberg equilibrium and did not differ significantly from those published in a population of UK "well" children (Mead et al., 1997). This was despite the difference in the ethnic mix of the 2 groups with Meads study having a non-Caucasian component of 2.4% and mine, for this study period, a self stated non-Caucasian component of 30% (Table 6.3).

Genotype	Frequency in PICU population n=142 (%)	Frequency in UK "control" population (%)*
A/A	64.1	59.6
A/O	34.5	35.8
O/O	1.4	4.6
A/O + O/O	35.9	40.4

A)

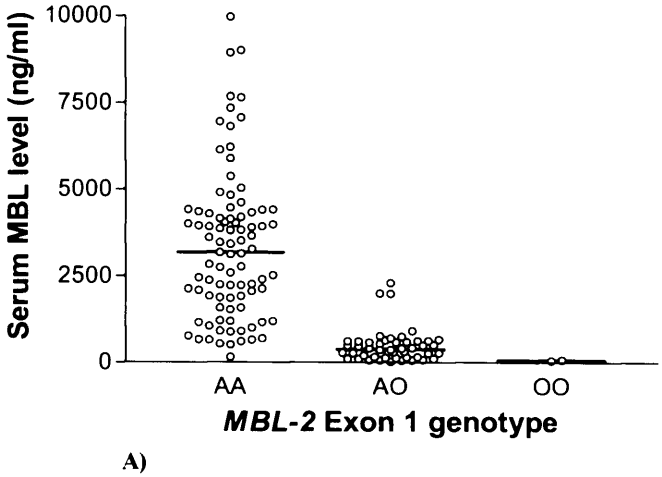
Allele	Frequency in PICU population n=142	Frequency in UK "control" population *
Wild type (A)	0.813	0.775
Codon 52 mutation (D)	0.060	0.066
Codon 54 mutation (B)	0.085	0.144
Codon 57 mutation (C)	0.042	0.015

B)

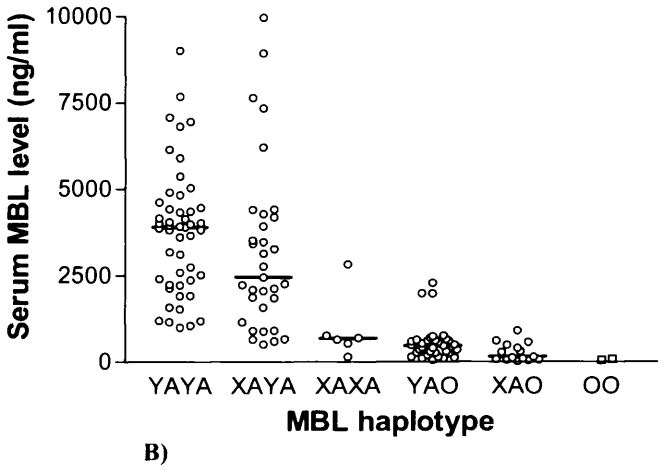
Table 6.3 Expected and observed distribution of MBL genotype (A) and allele (B) frequencies. The frequencies in the study population were not significantly different to those from a published UK control population *(Mead et al., 1997)

6.3.3 Relationship of MBL haplotype to serum MBL level

As expected, there was a significant correlation between serum MBL level and MBL genotype and haplotype ($p<0.001$ Kruskal Wallis). The highest protein levels were seen in patients with wild type structural alleles, and the lowest in individuals homozygous or compound heterozygous for structural mutations, and in heterozygotes with the X promoter mutation (Fig 6.1). MBL levels were higher in patients heterozygous for the D mutation compared to the the B and C variants. This finding has now also been demonstrated in the largest study to date in children followed up for the Avon Longitudinal Study of Parents and Children (ALSPAC) (personal communication Prof. M. Turner).



A)



B)

Figure 6.1 Relationship between serum MBL levels and A) *MBL-2* exon 1 genotype and B) MBL haplotype including X and Y promoter polymorphisms n=139. Serum levels in both cases are significantly related to genotype and haplotype (Kruskal-Wallis $P<0.0001$ for both).

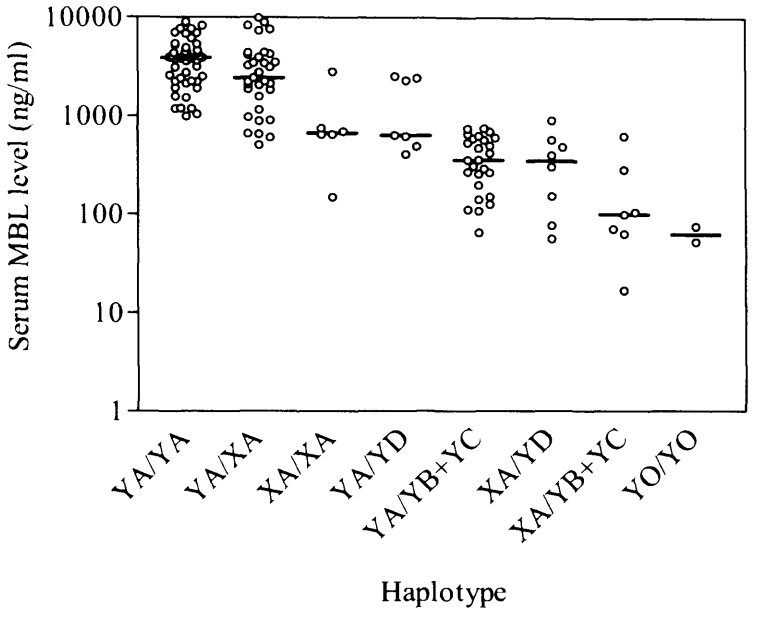


Figure 6.2 Relationship of serum MBL level to MBL haplotype for 139 children on PICU. Heterozygotes (A/O) have been further divided into those with a mutation at codon 52(D) and those with mutations at codon 54(B) and 57(C). Serum levels are significantly related to haplotype (Kruskal-Wallis $P<0.0001$). Bars represent median levels. 0=B+C+D

6.3.4 Relationship of MBL to age, gender, ethnic origin and C-reactive protein levels.

The data demonstrated in Fig 6.3 shows no correlation between age and serum MBL (Spearman linear correlation coefficient 0.02, p=0.83) but does demonstrates that even young babies can generate MBL levels of up to 9000 ng/ml in this clinical situation.

Ethnicity is an important determinant of MBL serum levels due to the difference in frequency of MBL gene mutations in various populations world wide (Turner, 1996). Overall in this PICU cohort there was no difference in MBL serum levels or proportions of variant genotypes between those *stated* Caucasian and non-Caucasian children (Fig 6.4). There was also no relationship of MBL serum levels to gender (data not shown).

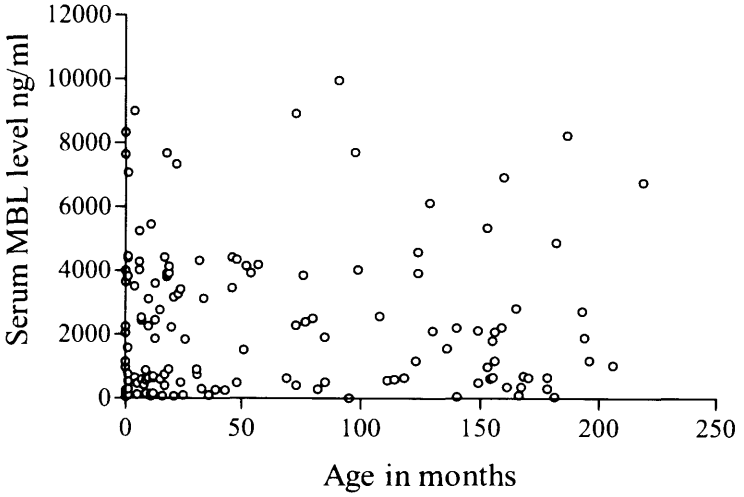


Figure 6.3 Relationship of serum MBL to age (n=139). There was no linear correlation between age and serum MBL (Spearman correlation coefficient 0.02, p=0.83). This data also demonstrates that even young babies can generate MBL levels of up to 9000 ng/ml in certain circumstances such as when in intensive care.

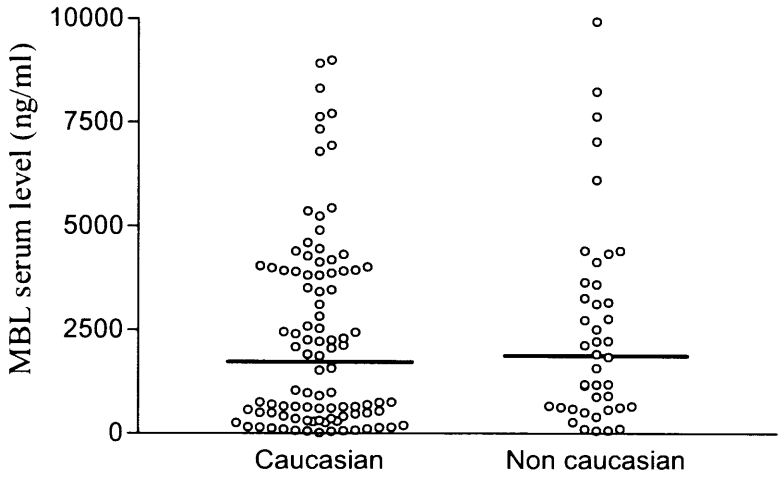


Figure 6.4 Relationship between serum MBL level and stated ethnicity in PICU patients (n=139).

No significant difference was seen in serum MBL levels of Caucasians and all non-Caucasian patients.

6.3.5 MBL variant alleles and low serum levels are associated with the development of SIRS

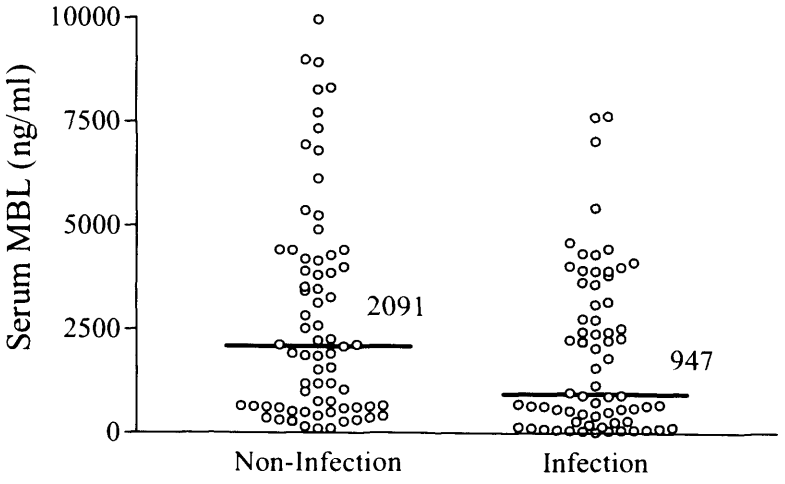
Fig. 6.5 shows both MBL serum levels and the proportion of cases with MBL-2 exon 1 mutations, by infective or non-infective reason for admission to PICU. MBL levels were significantly lower in those children admitted with infection ($p<0.028$, Mann-Whitney) and a trend was apparent between the proportion of children with a variant allele in whom infection had precipitated PICU admission compared to those without infection (43.5% vs.28.8%) ($p=0.06$ Chi squared, OR 1.9, 95% CI 0.95-2.02).

Analysis of the development of SIRS revealed significantly lower MBL serum levels ($p<0.0001$ Mann- Whitney) and a significantly higher proportion of variant alleles ($p=0.0003$ Chi squared OR 4.1, 95% CI 1.9-8.9, Fig. 6.6) in those children who developed SIRS compared to those who did not. This relationship remained after age, sex and ethnicity were accounted for. Further analyses of subgroups revealed that in both the 'non-infection' (Fig 6.7 A) and 'infection' sub-groups (Fig 6.7 B) the significantly increased odds of developing SIRS in association with an MBL variant allele remained ($p=0.019$ Chi sq, OR 3.49, 95% CI 1.19-10.1 and $p=0.02$ Chi sq, OR 4.06, 95% CI 1.18-14 respectively). Within both subgroups serum MBL levels were again significantly lower in those who developed SIRS than those who did not (without infection $p=0.05$, Mann- Whitney U and with infection $p=0.0014$, Mann- Whitney U)

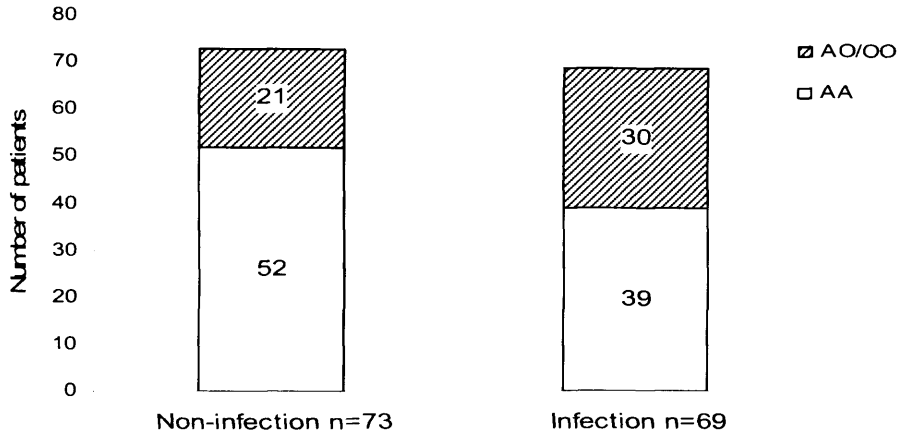
(Fig. 6.8 A&B).

As expected SIRS occurred more frequently in haplotypes associated with reduced MBL levels. Only 2 children were homozygous for an *MBL-2* exon 1 polymorphism, one D/D (52/52) and one B/C (52/57), both of whom developed SIRS. Interestingly 13/15 (87%) of patients heterozygous for an MBL exon 1 mutation associated with a

low expression promotor (XA/YO) developed SIRS and the risk of SIRS decreased with increasing prevalence of the A and Y alleles ($p=0.0079$, Chi squared, Table 6.4).

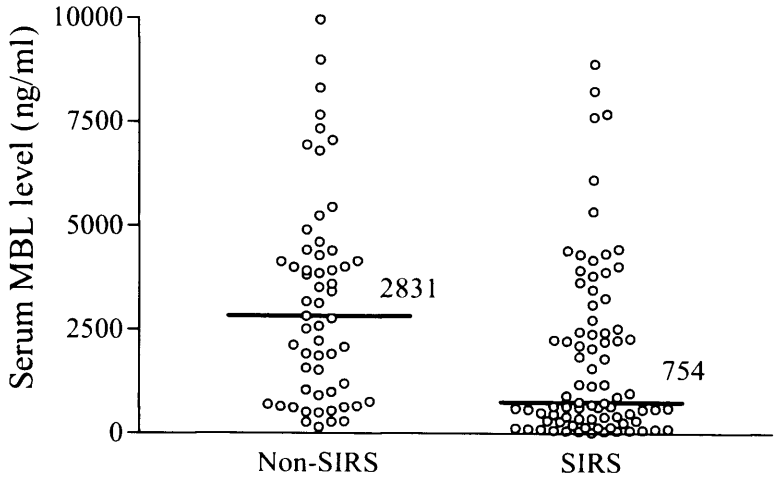


A)

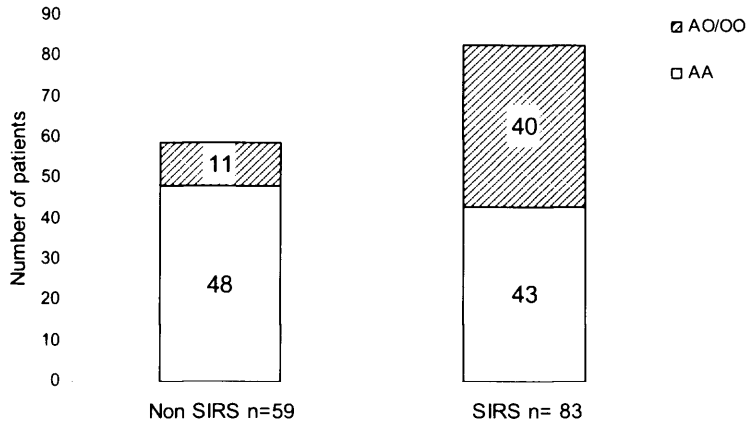


B)

Fig. 6.5 Relationship of MBL serum level (A) and *MBL-2* exon 1 genotype (B) to whether or not infection precipitated admission to PICU, n=142. **A)** MBL levels were significantly lower in those children admitted with infection ($p<0.028$, Mann-Whitney). Individual and median values are shown. **B)** A higher proportion of children with MBL variant alleles were admitted with infection although this did not reach significance ($p=0.06$ $\chi 2$, OR 1.9, 95% CI 0.95-2.02).

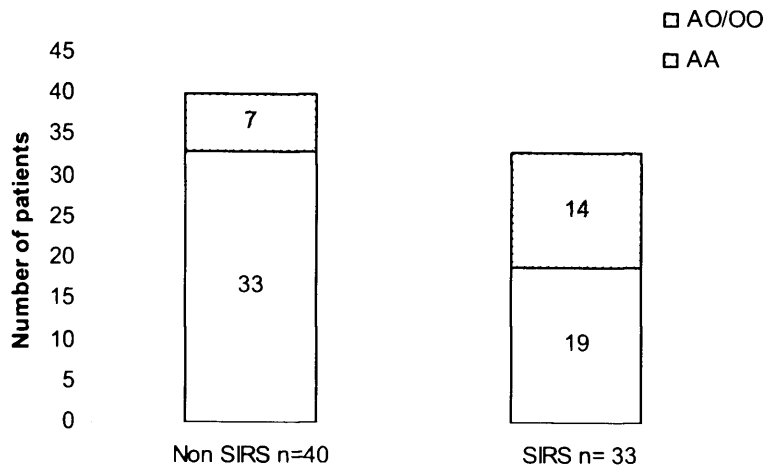


A)

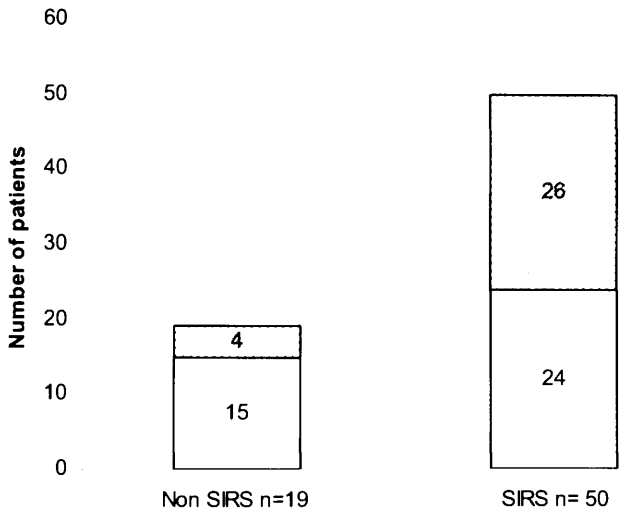


B)

Fig. 6.6 Relationship of MBL serum level (A) and *MBL-2* exon 1 genotype (B) to the early development of SIRS in children requiring intensive care, n=142. A) MBL levels are significantly lower in children who developed SIRS ($p<0.0001$, Mann-Whitney). Individual and median values are shown. **B)** A significantly higher proportion of children with MBL variant alleles developed SIRS ($p=0.003$ $\chi 2$, OR 4.1, 95% CI 1.9-8.9).

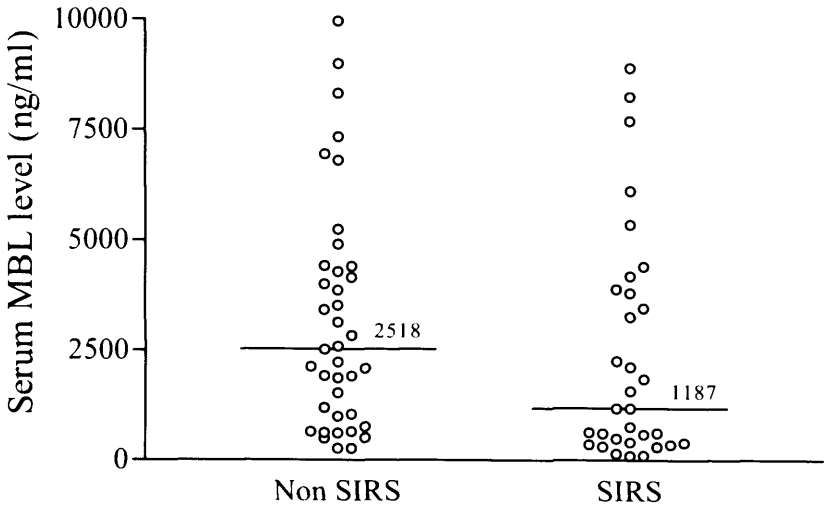


A) Patients admitted without infection

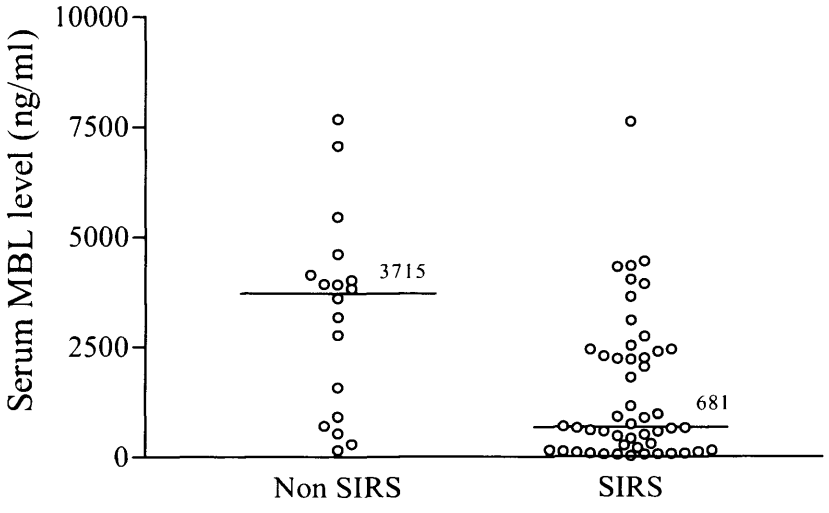


B) Patients admitted with infection

Fig. 6.7 Proportion of children who developed SIRS according to an admission diagnosis of non infection or infection. In both patient groups the presence of an MBL variant allele was still associated with the development of SIRS A) p=0.019 chi sq, OR 3.49, 95% CI 1.19-10.1 and B) p=0.02 chi sq, OR 4.06, 95% CI 1.18-14.



A) Patients admitted without infection



B) Patients admitted with infection

Fig. 6.8 MBL serum levels in children who developed SIRS according to admission diagnosis of infection or non infection. In both children admitted without and with infection MBL serum levels were lower in those patients who developed SIRS compared to those who did not develop SIRS (p=0.05 and p=0.0014, Mann-Whitney U, respectively).

Haplotype	SIRS n=83	Non SIRS n=59	Total n=142
YA/YA	21 (42%)	29 (58%)	50
YA/XA	18 (53%)	16 (47%)	34
XA/XA	3 (50%)	3 (50%)	6
YA/YO	25 (74%)	9 (26%)	34
XA/YO	13 (87%)	2 (13%)	15
YO/YO	2 (100%)	0 (0%)	2
Failed promotor genotype	1		1

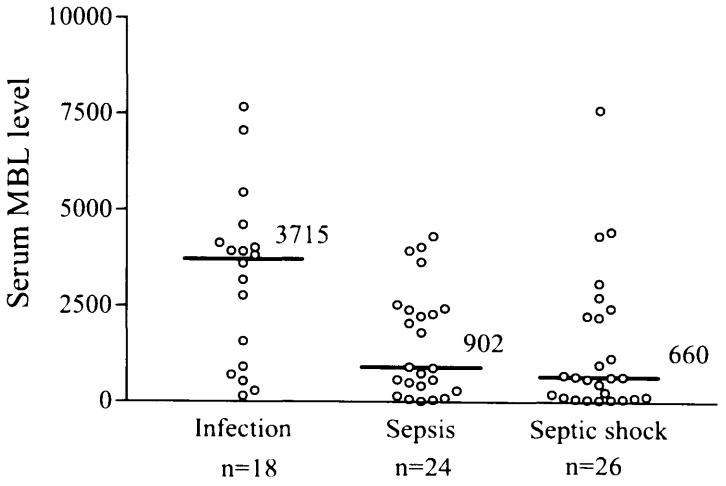
Table 6.4 Relationship of the development of SIRS to MBL short haplotype.

A significant correlation is seen between the increasing prevalence of A and Y alleles and the decreasing incidence of SIRS (p=0.0079, Chi squared)

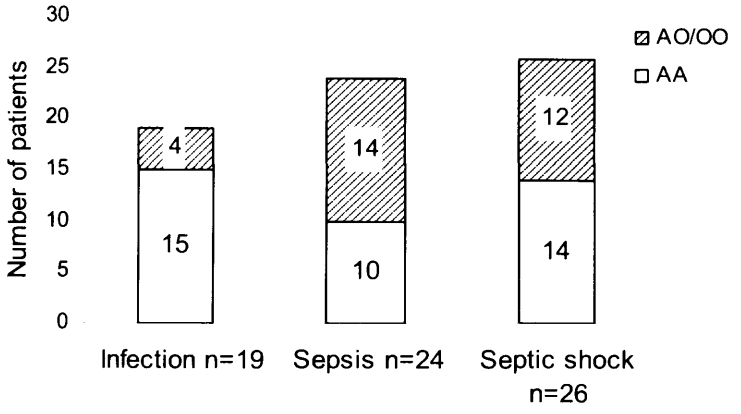
6.3.6 MBL variant alleles and low serum levels are associated with an increasing severity of sepsis

Median serum MBL levels decreased significantly with increasing severity of infection (p=0.005 Kruskal-Wallis) (Figure 6.9 A). This was even more striking in the first 100 patients analysed (Fidler et al., 2004).

In the whole cohort a significant association was seen between the severity of the systemic response to infection and the presence of an MBL variant allele (localized infection 4/19 vs. sepsis 14/24 vs. septic shock 12/26) (p=0.047, chi squared, Figure 6.9 B). When sepsis and septic shock are combined, as in some papers (Gordon et al., 2006) and compared to localized infection this significance increases (4/19 Vs 26/50 p=0.02 chi squared). In the first 100 patients analysed this relationship was again more striking (localized infection 2/15 vs. sepsis 10/19 vs. septic shock 12/16) (p=0.002 chi squared) (Fidler et al., 2004).



A)



B)

Figure 6.9 Relationship of MBL serum level (A) and *MBL-2* exon 1 genotype (B) to the severity of infection (n=142). **A)** MBL levels are significantly lower in those children admitted with sepsis and septic shock than those with infection alone (p=0.005 Kruskal-Wallis). Individual and median values are shown. **B)** A higher proportion of children with MBL variant alleles developed sepsis or septic shock (p=0.047 $\chi 2$).

6.3.7 The Relationship of MBL and C-reactive protein levels.

A weak inverse linear relationship was seen between CRP and MBL serum levels (Spearman r = -0.1747, p= 0.041) and a weakly positive linear relationship between serum CRP and the highest SOFA score (Spearman r =0.306, p= 0.0003), Figure 6.10. This may reflect that, in this cohort, patients with more severe sepsis and SIRS (and hence higher CRP levels) are more likely to be MBL deficient.

6.3.8 MBL and association with mortality and severity of illness in PICU patients

Lower levels of serum MBL were associated with higher on admission PIM scores (r-1.6, p=0.06, Spearman) and maximum SOFA scores (r-0.23, p=0.0078, Spearman) (Fig 6.11). Mortality for this cohort was 9% which was typical for this unit and considerably lower, as expected, than in adult intensive care studies (Simpson et al., 2005). Fatality rates were similar in the MBL sufficient and insufficient individuals. From Fig 6.12 it can be seen that those who died seemed to have low levels of MBL (due to polymorphisms) or higher than normal levels (>2500 ng/ml) probably reflecting an acute response to their illness. Only two patients who died had a "normal" MBL level. Eight of the 13 children who died were admitted with an infectious aetiology (1 enteroviral meningitis infection, 2 sepsis, 5 septic shock) and 5 without (3 head injuries, 1 post operative, 1 drowning). Analysis of MBL levels and survival within the "infectious" and "non-infectious" groups did not reveal any significant differences either.

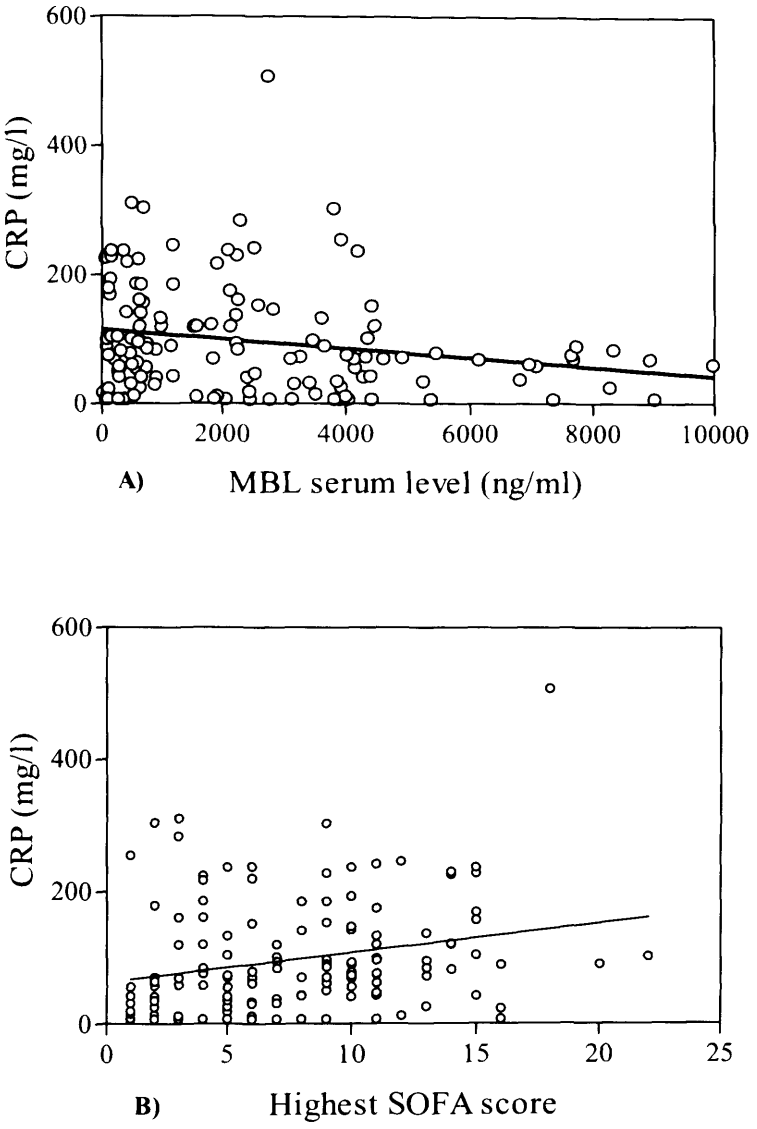


Fig 6.10 The relationship of C-reactive protein to A) serum MBL and B) highest SOFA score in PICU patients. A weak but significant inverse linear relationship is seen between serum CRP and MBL levels (Spearman r = -0.20, p= 0.019). A weak but positive linear relationship is seen between serum CRP and the highest SOFA score for the patient (Spearman r =0.306, p= 0.0003).This may reflect that in this cohort patients with the higher CRP's are those with MBL deficiency and more severe disease.

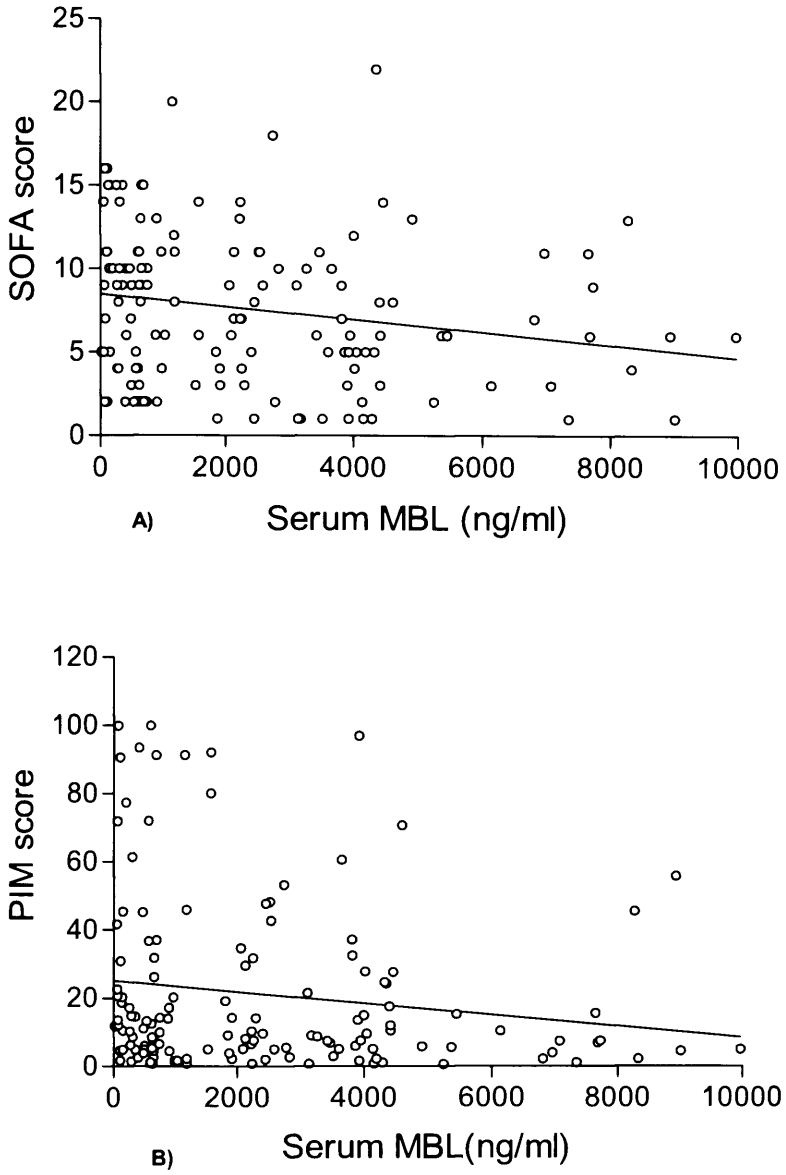


Fig 6.11 The relationship of serum MBL levels with PIM and SOFA scores

A weak inverse correlation between serum MBL and paediatric risk of mortality (PIM) and sequential organ failure assessment (SOFA) scores was apparent. Lower levels of serum MBL were associated with higher on admission PIM scores (r-1.6, p=0.06, Spearman) and maximum SOFA scores (r-0.23, p=0.0078, Spearman).

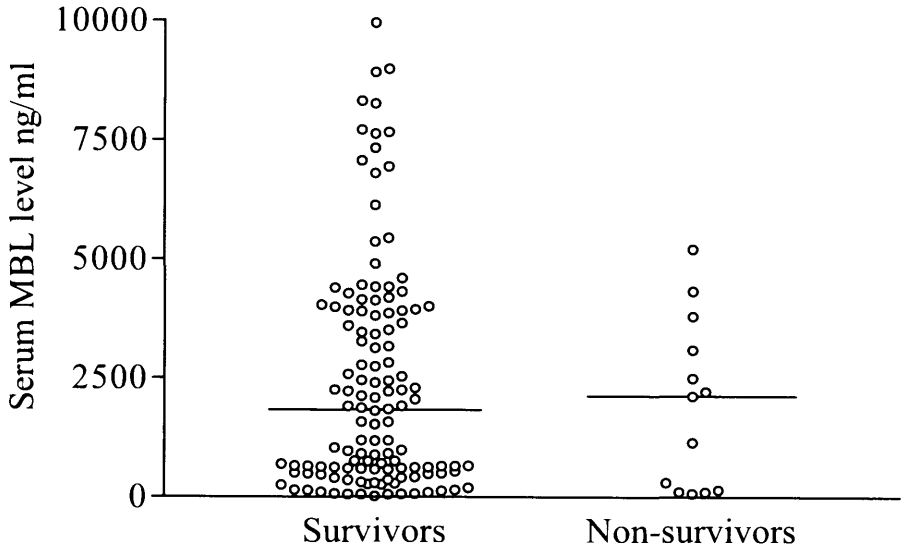


Fig 6.12 Serum MBL level in patients who died and in those who survived n=139.

There was no significant difference between these groups (p=0.48, Mann-Whitney test) although the number of patients who died is small n=13 (9%). The bold line demonstrates the median MBL level for each group.

6.3.9 The effect of MBL on the prevalence of the infecting microorganism

A wide spectrum of micro-organisms cause infection in intensive care patients. In this series of 69 children with an infectious insult precipitating admission the following organisms were thought to be causative; gram positive bacteria: 15 (21.7%), gram negative bacteria: 23 (33%), viruses 12 (17.4%), mycoplasma 2 (2.9%), fungi 0. No organism was isolated in 17 (24.6%) cases, probably due to the prior administration of antibiotics. Further identification of these organisms is shown in Table 6.5. Overall a trend was seen towards a causative organism being identified in those with an MBL variant allele although this did not reach significance ($p=0.07$ $\chi 2$ test). No association was seen with specific class of microorganism and MBL deficiency.

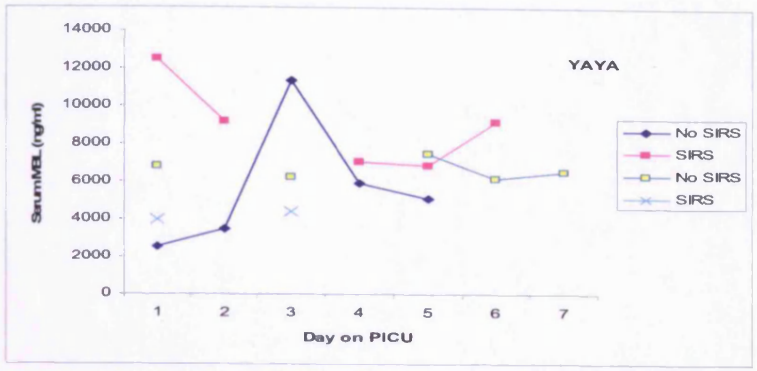
Infecting Microorganim	Number (%) of Cases n=69	Disease caused
Group A streptococcus	1 (1.4%)	Sepsis
Group B streptococcus	3 (4.3%)	Meningitis 1, sepsis 1, mixed 1
Viridans streptococcus	2 (2.9%)	Sepsis
Pneumococcus	4 (5.8%)	Meningitis 1, sepsis 1, mixed 2
Staphylococcus aureus	2 (2.9%) (1 MRSA)	Sepsis
Staphlococcus epidermidis	1 (1.4%)	Sepsis
"other" gram positive organisms	2 (2.9%) (1 enterococcus,1 bacillus)	Sepsis
Nisseria menigitidis	6 (8.7%)	Sepsis 5, meningitis 1
Pseudomonas	3 (4.3%)	Pneumonia 2, sepsis 1
Ecsherichae coli	2 (2.9%)	Sepsis
Klebsiella	3 (4.3%)	Sepsis
Haemophilus influenzae	5 (7.2%) (2 type b)	Epiglotitis 1, meningitis 1, sepsis 1, tracheitis 1, mixed 1
"other" gram negative rods	4 (5.8%)	Sepsis 3, pneumonia 1
Respiratory syncitial virus (RSV)	6 (8.7%)	Bronchiolitis 3, bronchiolitis and sepsis 3
Enterovirus	4 (5.8%)	Meningitis 1, myocarditis 3
Herpes simplex virus	1 (1.4%)	Encephalitis
Epstein Barr Virus	1 (1.4%)	Pneumonitis
Mycoplasma pneumoniae	2 (2.9%)	Pneumonia
Fungi	0 (0%)	
No organism isolated	17 (24.6%)	Infection 7, sepsis 10

Table 6.5 Table of disease causing organisms isolated from 69/142 cases admitted with infection to PICU. The spectrum of diseases is shown.

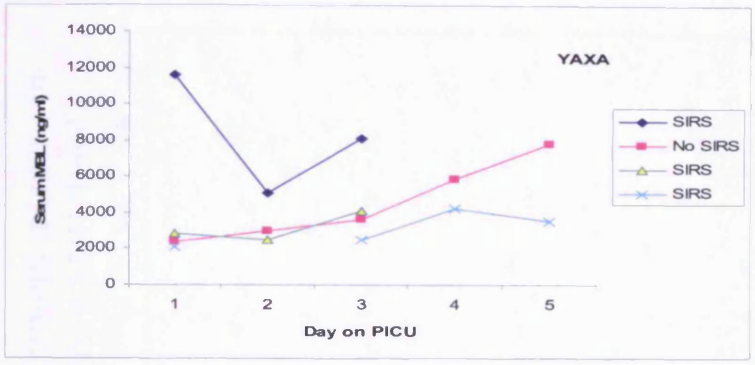
6.3.10 Serial serum MBL measurements

Serial measurements were performed in 11 children on PICU (Fig 6.13). Nine wild type individuals had marked variation in MBL levels with no obvious pattern seen in those who did or did not develop SIRS. The one child with genotype YA/O had the D (52) mutation associated with higher levels than normal for a heterozygote and interestingly although his levels were mainly insufficient he did generate a level of approximately 2000 ng/ml on admission.

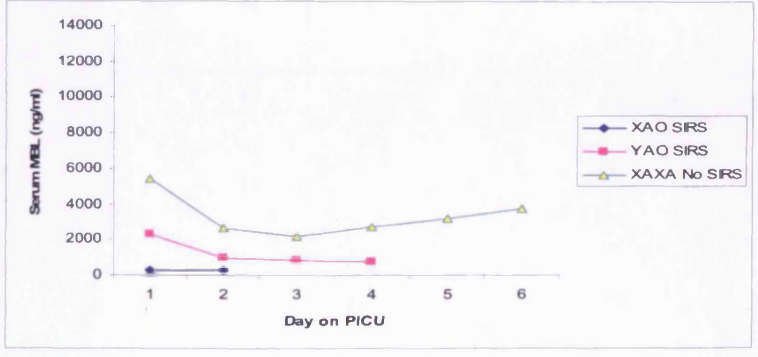
Only one patient was MBL insufficient with genotype XA/O. Like the children shown in chapter 3 (section 3.3.8) this child also did not raise his MBL level into the normal range despite having a substantial insult ie SIRS. Maximal increase in serum MBL are shown in Fig 6.14 where increases are similar, in order of magnitude, to those previously published (Thiel et al., 1992).



A)



B)



C)

Fig 6.13 Serial MBL levels in patients on PICU.

Data are shown for those with MBL genotype **A)** YAYA, **B)** YAXA and **C)** combined XAXA, YAO and XAO in patients with and without SIRS. In this small sample no significant relationship is seen between MBL genotype, the presence or not of SIRS and the ability to mount an acute phase response, however those with low producing genotypes produce lower, or no (XAO patient, graph C) responses.

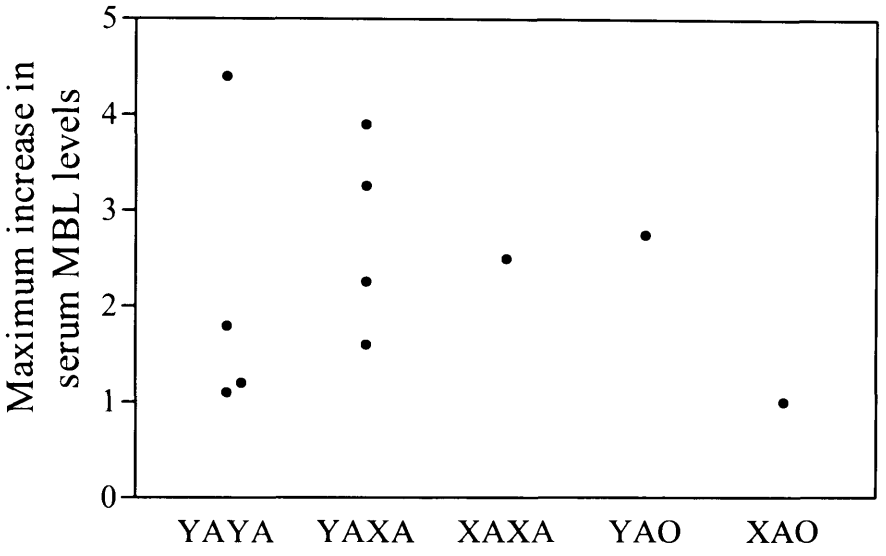


Fig 6.14 Relationship between MBL group and maximum increase in serum MBL level. Numbers are too small to comment on the relationship between genotype and maximum increment in serum MBL levels. The one child with genotype YA/O had the D (52) mutation associated with higher levels than normal for a heterozygote.

B) The effect of MBL on the production of cytokines by monocytes :

6.3.11 Confirmation of cytokine detection after whole blood stimulation with LPS

Fig 6.15 demonstrates the positions of lymphocytes, monocytes and other cells (mainly neutrophils) on forward and side scatter plots by FACS analysis. The monocytes were also identified by being the only group of cells to be CD14 positive (Fig 6.16). Demonstration that the "system" works is shown in Fig 6.17 with the production of TNF-α by CD14 positive monocytes in stimulated but not unstimulated cells. IgG1 PE controls are shown in both the stimulated and unstimulated experiments. Further demonstration of this is shown in Fig 6.18 where the stimulation of whole blood with both 1 and 50 ng of LPS resulted in significant IL1β production by CD14+ monocyte cells. The pattern of TNFα and IL-6 production was almost identical (data not shown).

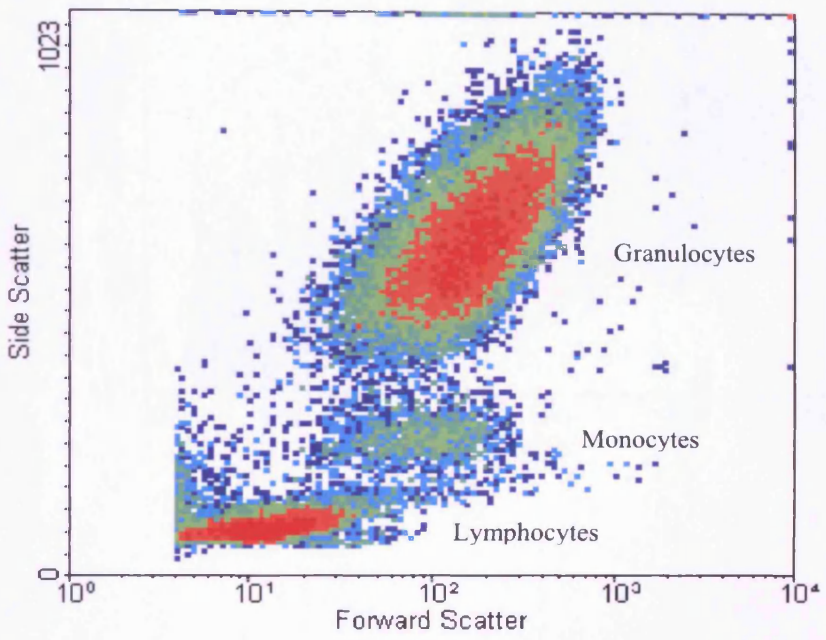
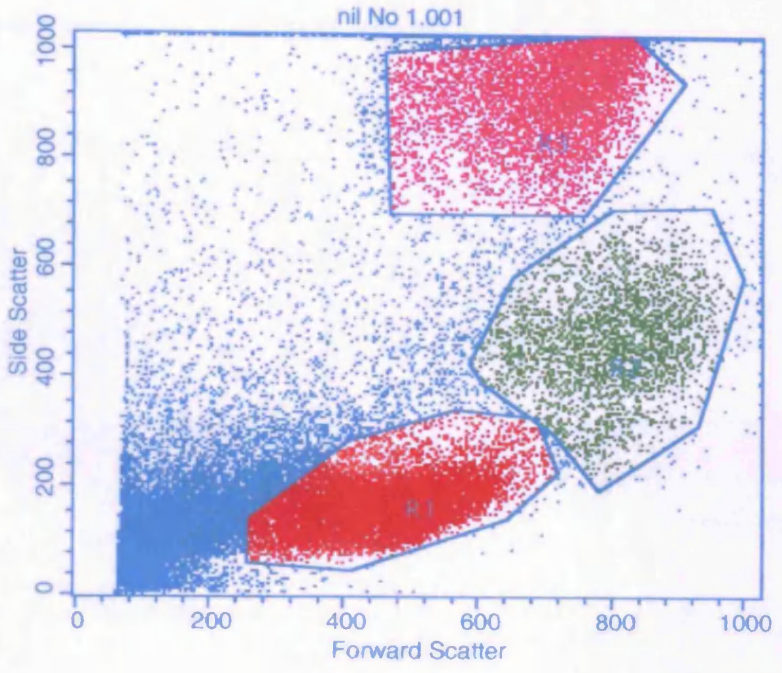
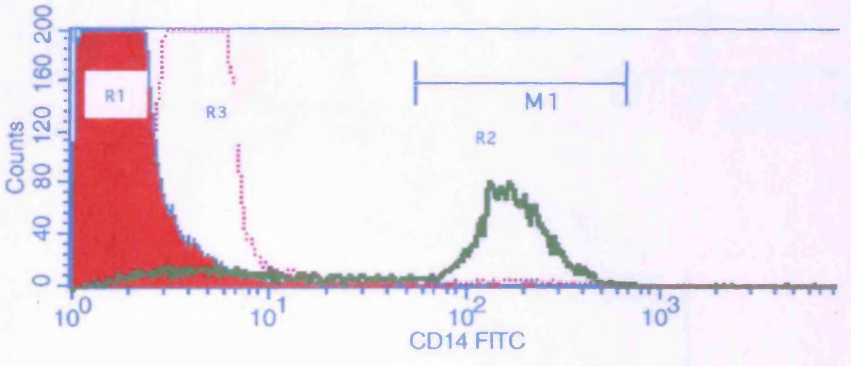


Figure 6.15 Flow cytometric profile of whole blood after lysis of red blood cells.

Anticoagulated whole blood was incubated with the appropriate monoclonal antibodies before lysis of red blood cells and fixation of remaining cells. The sample was then passed through the flow cytometer where each particle was assessed and plotted according to its size (forward scatter) and granularity (side scatter). Monocytes were recognisable as the smaller population of cells located between granulocytes and lymphocytes.



A)



B)

Fig 6.16 Identification of monocytes as the only cells to be positive for CD14.

A) shows the flow cytometric profile of whole blood (after lysis of red blood cells) with gates around each cell population. **B)** shows the same cells in histogram format and demonstrates that only the gated monocytes are CD14 positive.

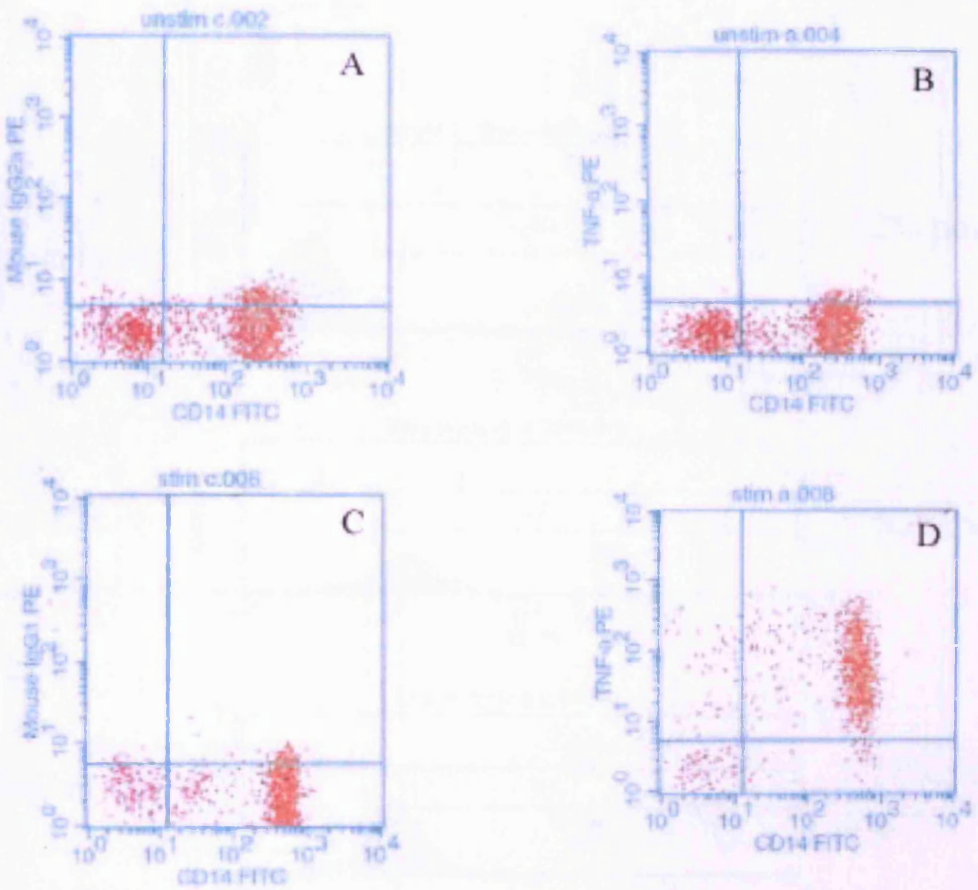


Fig 6.17 Production of TNF by monocytes after stimulation with LPS

Flow cytometer gain settings were adjusted to ensure that the negative controls were shown in the lower part of the quadrant. **A** and **B** are unstimulated cells, **C** and **D** are stimulated with LPS. Cells positive for both CD14 (FITC) and TNFα (PE) are clearly seen in the upper right quadrant (**D**). Staining of cells is specific for the cytokine, in this case TNFα, as can be seen with the PE isotype control (unstimulated **A**, stimulated **C**).

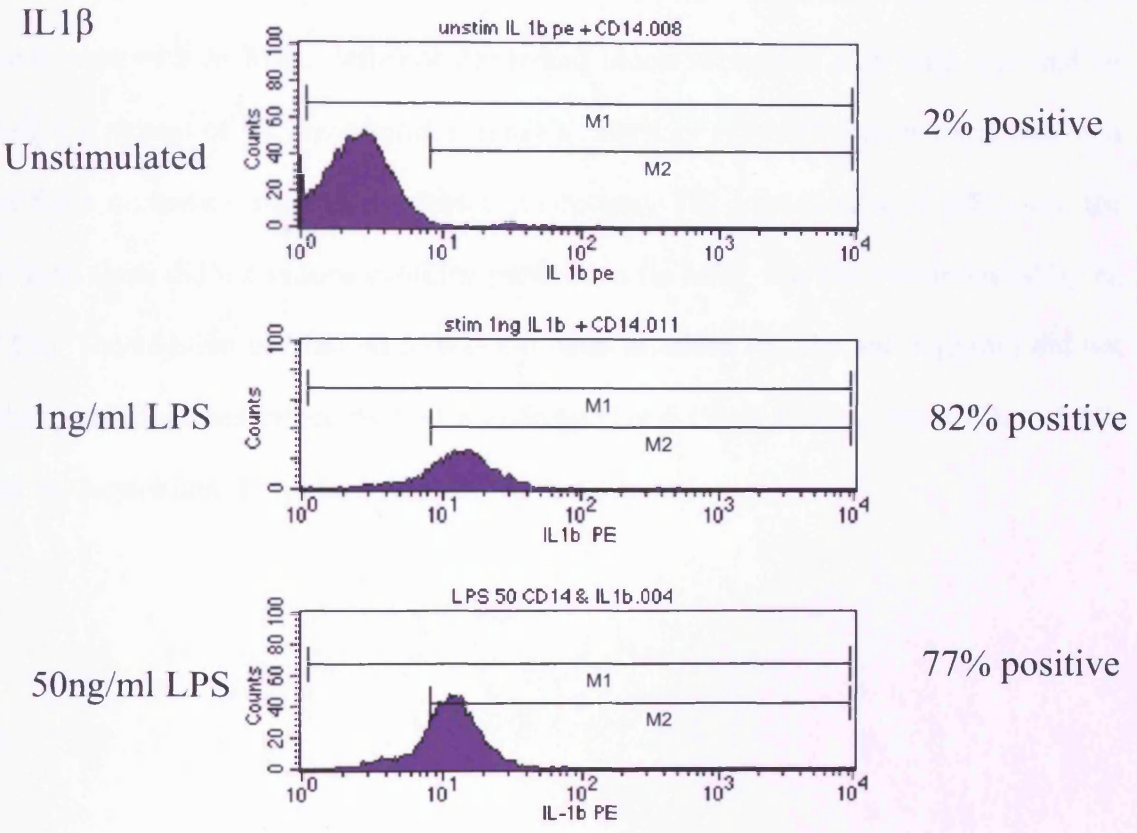
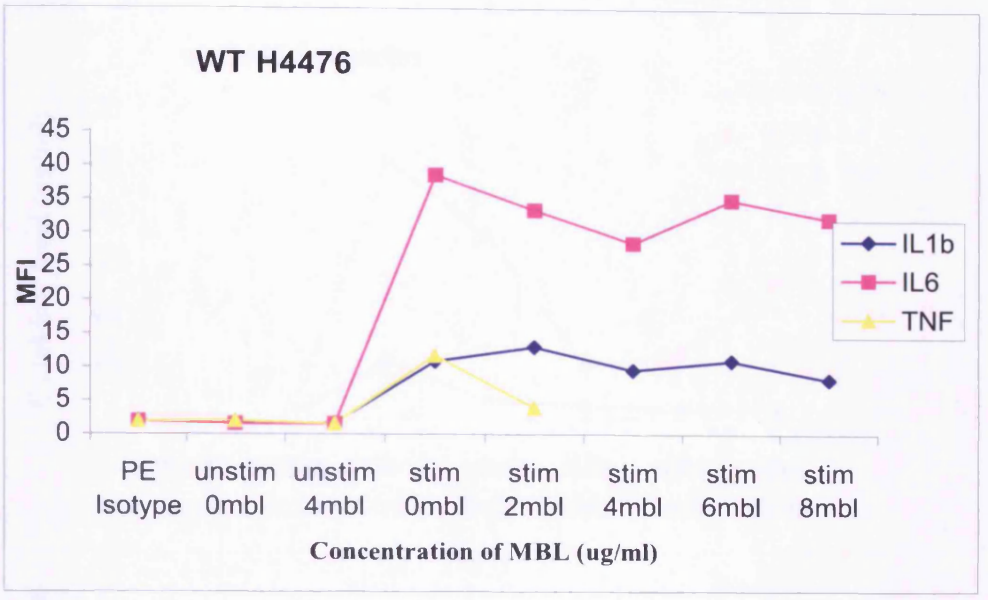


Fig 6.18 The influence of lipopolysaccharide (LPS) on CD14+ monocyte production of IL1-β. Whole blood was cultured with either 1 or 50 ng/ml of LPS in the presence of brefeldin A for 4 hours at 37°C with 5% CO2. The unstimulated control was incubated with culture medium and brefeldin only. CD14 surface staining followed by intracellular IL1-β cytokine staining was performed and cells analysed by FACS. The presence of LPS at both concentrations resulted in a significant production of IL1-β compared to the control sample. By convention 2% of the control population were deemed positive for the PE flurochrome (background fluorescence).

6.3.12 The influence of MBL on IL1β, TNFα and IL-6 production by monocytes from an MBL deficient donor

Having demonstrated that cytokine production could be readily assessed following LPS stimulation in whole blood, the effect of MBL could be explored. Experiments were performed with an MBL deficient donor and blood stimulated with wild type and an isogenic mutant of *N. meningitides* (*cpD*-). Fig 6.19 A and B demonstrates that both of these organisms stimulate cytokine production. The introduction of MBL into the system alone did not induce cytokine production (ie MBL was not contaminated by eg LPS). The addition of MBL at 5 different concentrations (0,2,4,6 and 8 μg/ml) did not show any consistent effect, by FACS analysis (Fig 6.19) or ELISA (Fig 6.20), of MBL on the 3 cytokines, IL1β, IL-6 and TNFα., investigated.



A)

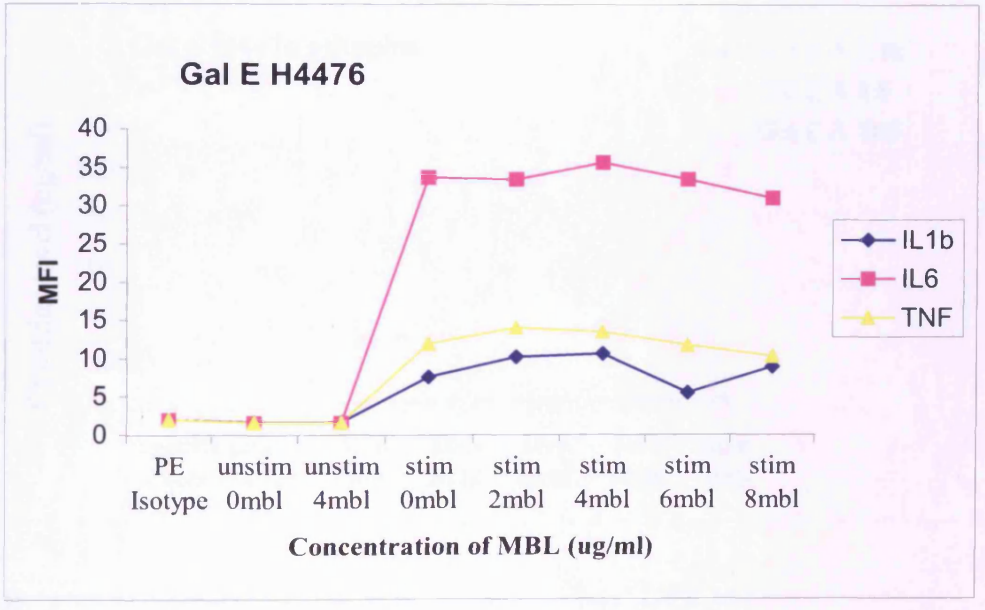
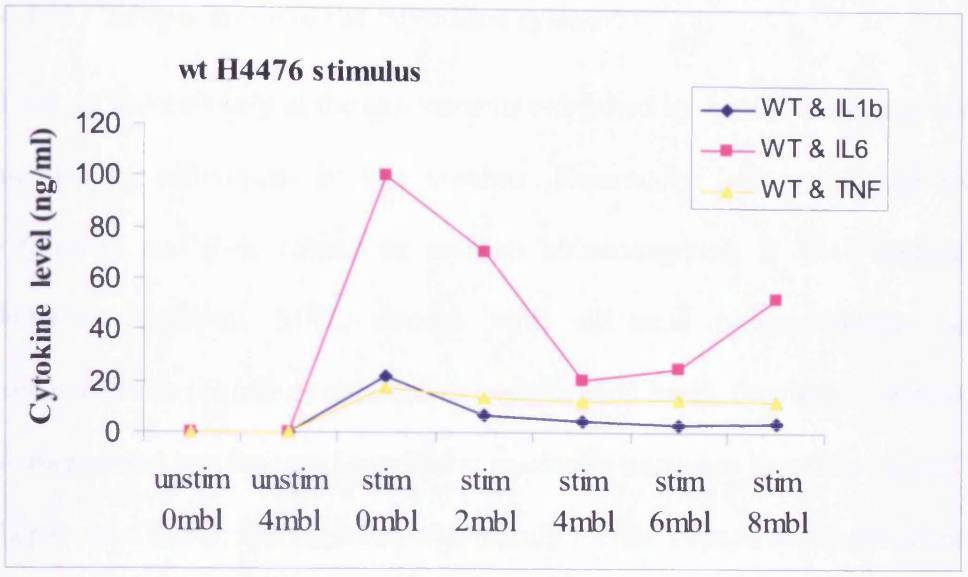
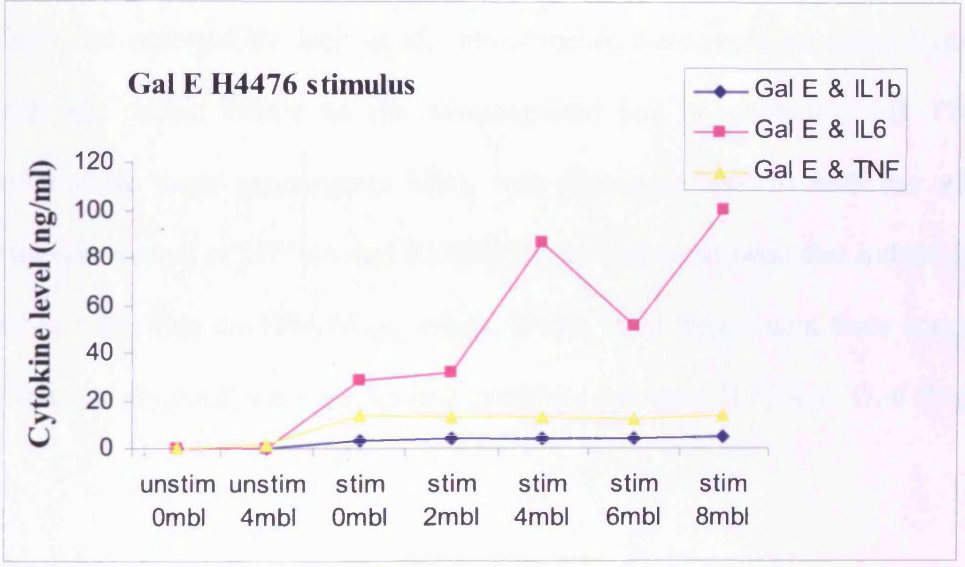


Fig 6.19 The influence of MBL on IL1β, TNFα and IL-6 production by monocytes from an MBL deficient donor: analysis by flow cytometry. No significant difference was seen in cytokine production in the presence of varying concentrations of MBL.

MFI = median fluorescent intensity



A)



B)

Fig 6.20 The influence of MBL on IL1β, TNFα and IL-6 production by monocytes from an MBL deficient donor: analysis by ELISA. No clear pattern of the effect of MBL was seen for either the wild type or gal E mutant of H4476.

6.3.13 Changes made to the "cytokine system"

Looking more closely at the experiments published by Jack et al and my own revealed a number of differences in our systems. Essentially Jack et al had used different organisms and their respective mutants (*N.meningitidis* B 1940 instead of Type B H44/76), different MBL donors with different polymorphisms and different anticoagulants (citrate as opposed to heparin used here). Previously published work has demonstrated that bacterial survival is markedly increased in citrated blood compared to heparinised blood, although this was mainly for the Type A *meningococcus* (Ison et al., 1995).

In order to determine if any of these factors contributed to the difference in findings from that reported by Jack et al, experiments were repeated using a different MBL deficient donor, citrate as the anticoagulant and *N.meningitidis* B 1940. Prior to performing these experiments MBL was shown to bind to both the wild type and isogenic mutant of H77/46 and B1940 (Fig 6.21) and showed that indeed B1940 bound more MBL than the H44/76 organism. In this latter experiment three concentrations of MBL (0,1,8 μg/ml) were used and 2 cytokines assessed; IL1β and IL-6 (Figure 6.22).

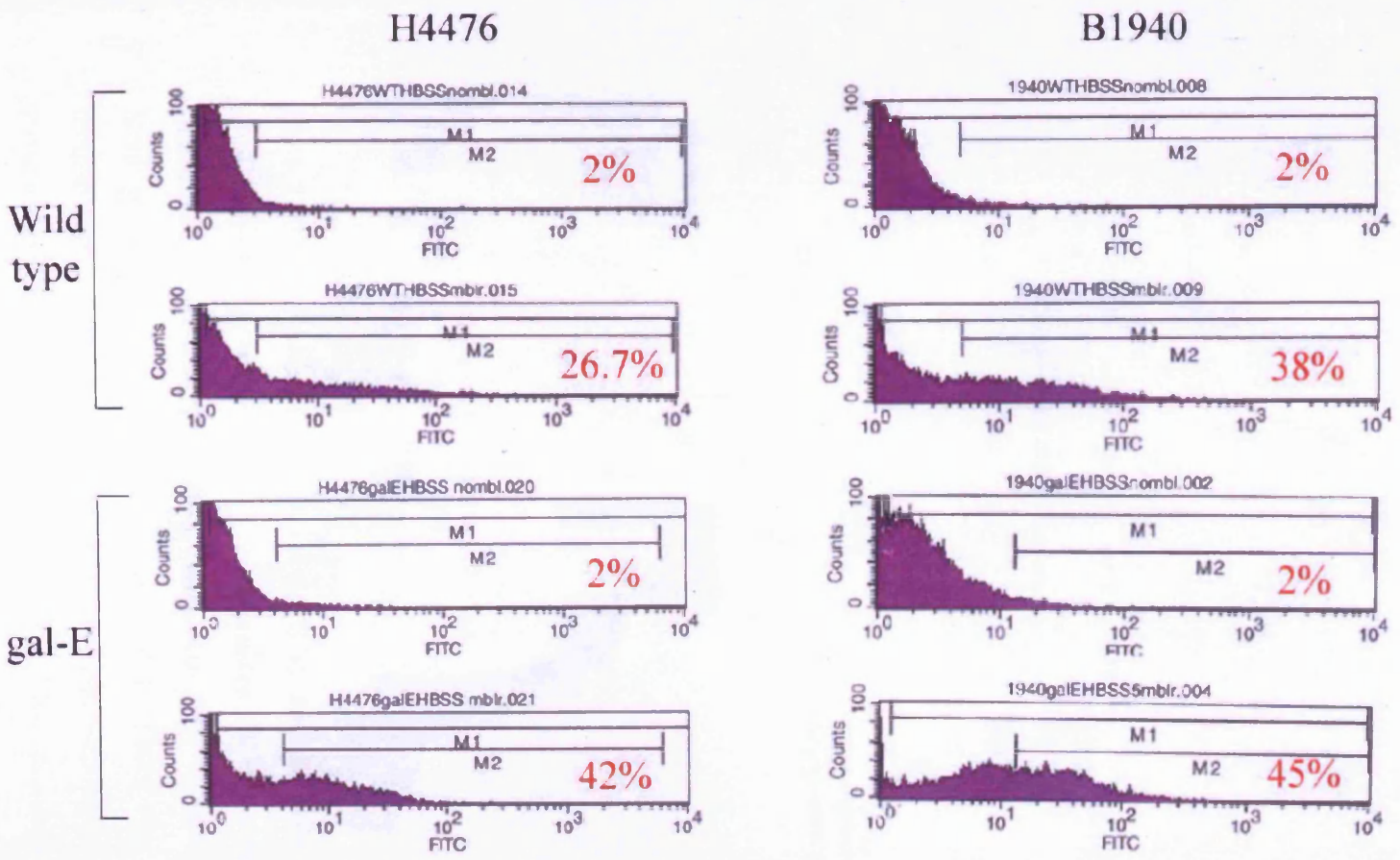
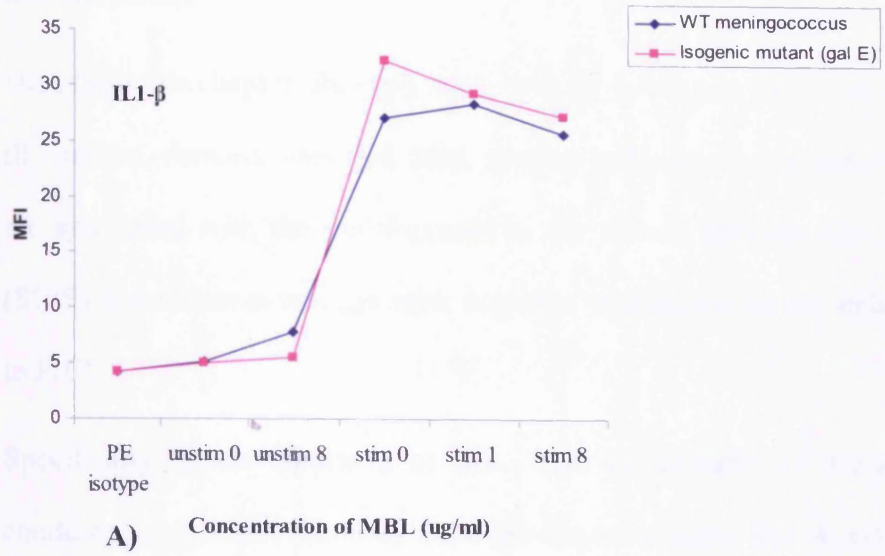


Fig 6.21 MBL binding to the wild type and *gal E* isogenic mutant of meningococci H 7746 and B 1940.
The top two histograms are wild type organisms and the bottom two are the gal E isogenic mutants. The percentage of cells positive for FITC are shown in red. By convention the negative control in each case, i.e. without MBL, was set at 2%. The histograms demonstate that MBL binds more to the *gal E* than WT for each organism and that indeed B1940 does bind MBL more than H7644.



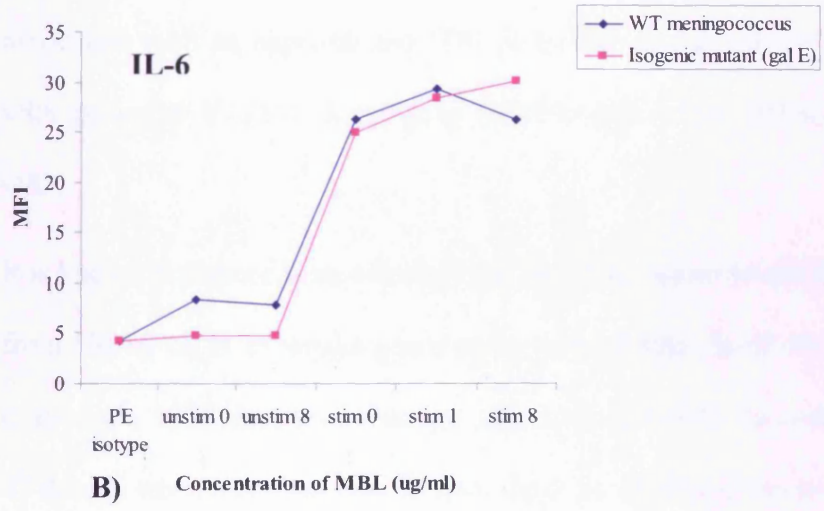


Fig 6.22 The influence of MBL on A) IL1β and B) IL-6 production by monocytes after stimulation with a different organism, B1960 meningogcoccus. Whole blood was preincubated with or without MBL and then stimulated with either WT B1940 meningococcus or an isogenic mutant, gal E. There was no significant increase in median fluorescent intensity (MFI) of the unstimulated cells with MBL compared to those without MBL. The addition of 1μg/mL MBL showed a small increase in IL1β and IL-6 which was abrogated by 8μg/mL MBL. This was seen in the wild type organism only.

6.4 Discussion

Data from this chapter, the only study to date looking at the effect of MBL in critically ill children, demonstrates that MBL genetic polymorphisms and reduced serum levels are associated with the development of the severe inflammatory response syndrome (SIRS) and a more severe systemic response to infection within 48 hours of presentation to PICU.

Specifically the development of SIRS was independent of the patient's underlying condition, age, sex or ethnicity although the association was strongest in those with an infective aetiology. The importance of MBL genotype in the development of SIRS is underlined by the observation that possession of the wildtype MBL haplotype is associated with an approximate 50% *reduction* in the risk of SIRS with 42% of those with genotype YA/YA developing SIRS compared to 100% of those with genotype O/O.

It is known that there is an effect of age on MBL serum levels in health. Levels increase from 500 ng/ml at 25 weeks gestation to normal adult levels by 20 weeks post full-term (Lau et al., 1995) and then seem to stay approximately the same from the 2nd until the 4th decade when a decline commences (Ip et al., 2004). In disease states the effect of age on MBL serum levels is not known and therefore this data also provides evidence that even young babies can generate MBL levels of up to 9000 ng/ml in certain circumstances such as critical illness. No sex differences in MBL levels have been described. MBL variant alleles are known to occur at different frequencies in different populations (see Introduction section 1.4.4) but surprisingly non-caucasians (self stated) were not overrepresented in those with MBL variant alleles. Despite the high non-Caucasian component (30%) of this cohort, due to the central London location and overseas referral patterns, ethnicity was not found to be associated with development of

SIRS or severity of sepsis suggesting no other ethnicity related factor was a confounding variable in the link between MBL deficiency and SIRS.

Whilst this present study was under review for publication (Fidler et al., 2004), Garred and colleagues in Denmark published a similar study on the role of MBL in critical illness in adults (Garred and Madsen, 2004). 272 adult Danish Caucasian intensive care patients with SIRS were compared to a control group of 190 blood donors and 60 hospital staff. The frequency of MBL variants in both groups was similar. However, there were significantly more MBL variant alleles in the infection-related reason for admission group when compared to the non-infectious reasons for admission. The severity of sepsis was linearly related to the frequency of MBL variant alleles (and inversely related to serum MBL levels) and the risk of mortality rose with the number of MBL variant alleles, homozygotes had a 2.3 relative risk (C.I. 1.19-4.48) for in-hospital death compared to wild-type. Considering the differences in the patients studied, there is remarkable concordance between the essential findings presented here and those of the Danish group. Both studies show an increasing over-representation of variant MBL alleles with increased severity of sepsis, although the ratio of WT/variant alleles is more dramatic in children. The results presented in this chapter show a strong relationship between MBL variants and the development of SIRS in the first 48 hours on ICU. These findings contrast with the Danish study in which MBL variants occurred with the same frequency amongst SIRS patients admitted to the ICU and healthy controls. Several possible explanations exist for these differences. This study is capturing the risk of developing early SIRS whilst fully supported on intensive care – which will include more cases than those with established SIRS on admission. The difference might also reflect the stringent criteria applied for a diagnosis of SIRS (with exclusion of the respiratory rate as a criterion), or differences between the control populations in the two

studies (here ICU cases without SIRS and in the Danish study healthy blood donors and laboratory staff). A remaining possibility is that these findings may reflect differences in the pathogenesis of critical illness between children and adults. One example of this is the high proportion of critically ill children who have no major pre-existing morbidity - a situation that will have been refined by our exclusion criteria. In contrast, the adult study reflects a typical pattern with the majority of cases having very significant pre-morbidity. There may also be differences in the referral patterns to ICU between children and adults with children being admitted earlier in the course of their illness. Finally, perhaps children and adults also differ in the contribution of the various mechanisms leading to organ dysfunction in SIRS. For example, MBL deficiency is known to be protective against ischaemia-reperfusion injury which may play a greater role in adults (Jordan et al., 2001). The Danish study also demonstrated an increased risk of death with MBL variants and SIRS. This could not be directly investigated in the paediatric cohort because of the low numbers of deaths.

In 2005 Sutherland and collegues also confirmed the association between MBL deficiency (and other innate immunity polymorphisms in CD14, and Toll Like Receptor -2) and an increased prevalence of positive blood cultures and sepsis in a cohort of adult Caucasian patients admitted to a mixed intensive care unit (Sutherland et al., 2005). In this study no association was seen with septic shock or risk of death. Again, differences in case mix may be important. Lastly 174 white adult patients with severe sepsis or septic shock were recruited in a prospective multicenter study across eight intensive care units in the South of England, UK. Genotype and haplotype frequencies were compared between normal population controls and patients, and between survivors and nonsurvivors. MBL polymorphisms (A/O or O/O) were significantly more common in

the patients with severe sepsis and septic shock than in normal healthy adults (54.6% vs. 39.7%, P = 0.001) (Gordon et al., 2006).

All of these studies lend support to the findings presented in this chapter that MBL deficiency does indeed increase the risk of SIRS or severe sepsis in paediatric patients admitted to intensive care.

A key question is whether MBL deficiency modulates the initial insult, the subsequent response to this insult or both. For example MBL deficiency seems to increase susceptibility to infection (Summerfield et al., 1997) but could also alter the host's response to micro-organisms and/or microbial products, potentially increasing the severity of illness. MBL deficiency could also have an effect on the development of SIRS remote from the primary insult. A central observation from this study points towards the latter as the likely explanation as a similar effect exists in trauma and post-operative cases when the role of MBL in opsonophagocytosis would appear to be of little consequence compared to the infection cases.

The reason for the observed effect of MBL on the development of SIRS remains unclear. It is thought that the principal role of MBL is to activate complement and this can occur even in the presence of an otherwise intact complement system (Brandtzaeg et al., 1996). The role of complement in critically ill patients is complex. Complement activation invariably occurs in patients with sepsis and following major surgery. For example, Brandtzaeg and colleagues have demonstrated marked complement activation in patients with meningococcal sepsis (Brandtzaeg et al., 1996). There are numerous pathways by which complement activation can lead to enhanced inflammation and therefore one might postulate that in fact a low MBL level may actually be beneficial as it may reduce inflammation and the development of SIRS. However a novel mechanism by which MBL could influence the development of SIRS is through a direct effect on

pro-inflammatory cytokine production. In a human whole blood model, high levels of MBL were observed to inhibit TNFα, IL-6 and IL-1β release from monocytes (Jack et al., 2001b). It is assumed that in this case MBL is operating through a receptor expressed, but as yet unidentified, on monocytes. MBL levels in patients presented in this chapter and those observed in the Danish study, were in a range which has been shown to inhibit cytokine production *in vitro* (Jack et al., 2001b).

Sprong *et al* have recently shown that low levels of binding, below the detection limits of conventional flow cytometric techniques, were still sufficient to modulate the inflammatory response. This involved a different model whereby human peripheral blood mononuclear cells (PBMC's) were stimulated with lipopolysaccahride (LPS) + or LPS- meningococci in a system with no exogenous complement. Here only lower concentrations of MBL were used (0.75-2500 ng/ml), different cytokines were assessed (IL-1β, IL- 6, IL-10, TNF-α and IFN-γ) and different results were obtained. The main findings were that MBL (<2500 ng/ml) significantly augmented IL-1β production after stimulation with both LPS+ and LPS- meningococci in a dose dependent fashion and also enhanced IL-10 production by LPS- meningococci. In contrast the production of IL- 6, TNF-α and IFN-γ was unaffected. Thus, even in the presence of pathogens to which the protein is thought to bind poorly, there may be a significant effect on inflammation, an often integral part of the disease process.

Whatever the mechanism underlying the influence of MBL on the severity of sepsis and the development of SIRS, this observation could prove to be clinically important. Firstly, considerable effort is currently being invested in analyses of risk stratification to identify patients likely to have the most severe patterns of illness. As this study indicates, it is possible to evaluate the host MBL status by partial genotyping (i.e. structural gene variants alone, Fig 6.1A), extended genotyping (to include the X/Y

promoter variant, Fig 6.1B) or by phenotypic assays of MBL protein levels (Fig 6.9). All three approaches showed remarkable concordance, therefore a simple serum test may provide an indicator of susceptibility to the development of SIRS. MBL is currently being prepared for clinical use and has been used in one patient with Cystic Fibrosis and end stage lung disease (Garred et al., 2002). We need to understand more clearly the costs and benefits of such therapy during specific episodes of critical illness but this study raises the possibility that judicious use of MBL in MBL deficient patients may lead to a reduction in the number that develop SIRS or severe sepsis/ septic shock.

This study provides data that contributes to the insight into the pathogenesis of SIRS & sepsis and may provide a novel therapeutic avenue for the treatment of critically ill children.

CHAPTER 7

The effect of cytokine polymorphisms and endotoxin antibodies on the role of MBL in inflammation

7.1 Introduction

Work presented in the previous chapter (chapter 6) has demonstrated the association between MBL deficiency, at both a genotypic and phenotypic level, with the development of SIRS and an increased severity of sepsis.

Gene association studies have to be regarded with caution due to a multitude of false positive associations (Sutherland & Russell 2005). These include linkage disequilibrium with an unknown candidate gene and confounding variables such as ethnic differences in both genotype frequencies and disease prevalence such that they are linked independently of the polymorphism. Despite these limitations, genetic associations that are found in large numbers and are repeatable in different situations are likely to provide clinicians with potential tools for the early assessment of risk in specific patient groups. This may allow more accurate prognosis, early intervention and treatment of high risk individuals and better analysis of risk benefit effects for low risk patients.

Genetic Polymorphisms

Over the last decade many studies have found associations between polymorphisms in genes that control levels of proteins in the innate immune system, and susceptibility to, and severity of, critical illness. However results from these studies are complex. It is likely that it is the combined effect of many of these polymorphisms that will ultimately

determine the host's response to an inflammatory challenge. Additionally the actual serum level of a protein or cytokine, and the timing of various responses at different points in the illness, may well be critical (Waage et al. 1989). This is highlighted in a recent study where the A allele of the IL-10 -1082 promoter polymorphisms was associated with *susceptibility* to severe sepsis, but once sepsis was established then presence of the G allele was associated with higher IL-10 levels and mortality (Stanilova et al., 2006).

Analysis of this published literature identified a number of key serum factors that may play a role in an individual's susceptibility to the development of SIRS and severe sepsis. These include polymorphisms in the tumour necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin 10 (IL-10), angiotensin converting enzyme (ACE) and plasma activator inhibitor 1 (PAI-1) genes as well as antibodies to endotoxin.

Tumour necrosis factor- alpha

TNF-α is an important cytokine associated with the development of systemic inflammation. High circulating levels have been associated with greater mortality rates in sepsis (Damas et al. 1989;Debets et al. 1989;Nadel et al. 1996). The gene encoding TNF-α is found within the MHC on chromosome 6. A polymorphism at position -308 in the promoter region of this gene has been identified, in which a G in the common allele is substituted for an A in the uncommon allele (found in approximately 30% of the Caucasian population). Although there are some conflicting studies, it seems overall, that the A allele is associated with higher constitutive and inducible TNF-α levels. This may be associated with adverse outcomes (Holmes et al., 2003) (Nadel, et al., 1996).

Interleukin-6

IL-6 is another pro-inflammatory cytokine with a short systemic half life, levels of which are also regulated by a number of factors, the most important of which is a polymorphism at position -174 in the promoter region where a single nucleotide G is substituted for a C. Higher levels of IL-6 have been associated with severity of sepsis and levels above 1000 pg/ml have been associated with increased mortality (Damas et al., 1992). Conflict in the literature relates to which allele causes the highest levels of IL-6, which may be age or disease related. In newborns the CC genotype was associated with higher IL-6 levels but no effect was seen in *healthy* patients (Kilpinen et al., 2001). In adults with abdominal aortic aneurysms, again the CC genotype was associated with higher levels (Jones et al., 2001). However other studies looking at patients post-cardiac bypass have found higher levels associated with the GG genotype (Burzotta et al., 2001) and in the patient group most similar to that described in this chapter (post-operative children on *cardiac* intensive care at GOSH) the GG genotype was associated with highest IL-6 levels (Allen M.L. personal communication).

Interleukin-10

IL-10 is an anti-inflammatory cytokine, levels of which may be critical in the modulation of the pro-inflammatory / anti-inflammatory balance. An excess of IL-10 however has been reported to induce immunosuppression in patients with sepsis, inflammation or post- cardiac surgery (Allen et al., 2006; Gomez-Jimenez et al., 1995; Lyons et al., 1997). Inter-individual differences in IL-10 levels are thought to be due to a number of polymorphisms in the promoter region of the IL-10 gene which is found on chromosome 1. These include nucleotide substitutions at positions -1082 (A/G), -819 (C/T) and -592 (C/A) of which -1082 has been the one most associated with severity of infection (Schaaf et al. 2003;Shu et al. 2003) and therefore chosen for study here.

Plasminogen Activator Inhibitor-1

Plasminogen Activator Inhibitor 1 (PAI-1) is released from endothelial and liver cells and inhibits plasminogen activators thus inhibiting fibrinolysis. High levels of PAI-1 thus result in a pro-thrombotic tendency, which may be detrimental in certain conditions such as sepsis and SIRS which may also have a pro-thrombotic potential. A polymorphism in the promoter region of PAI-1, involving an insertion or deletion of a guanine (G) base, has been described where possession of the 4 G allele results in 6 times the production of PAI-1. The 4 G allele has been associated with severity of disease outcome in severely injured patients (Menges et al. 2001) and in sepsis, specifically meningococcal sepsis (Haralambous et al. 2003; Kornelisse et al. 1996; Menges et al., 2001).

Angiotensin converting enzyme

Increased activity of the renin-angiotensin systems in human tissue drives a pro-inflammatory response (Weber et al. 2003). In such systems angiotensin converting enzyme (ACE) is not only an important enzyme for the generation of angiotensin II from angiotensin I but also degrades bradykinin. Both these molecules have been shown to have many cellular effects (Marshall et al. 2002). ACE inhibitors reduce the IL-6 response after coronary artery bypass graft surgery (CABG) and myocardial cell dysfunction (cardiac failure) (AIRE Study Investigators 1993) and are one of the ways in which this medication could be operating. It could be postulated therefore that low ACE levels could also confer an anti-inflammatory state. ACE is encoded by one of two variants of the ACE gene. One carries an insertion of 287 base pairs and is termed "I", the other does not and is called "D". The longer I allele is associated with lower enzyme activity which is beneficial in a number of circumstances. A number of studies have shown that presence of the I allele is associated with enhanced endurance performance to intense exercise (Woods, et al., 2000) and enhanced mechanical efficiency in trained

muscle (Montgomery et al., 1997). The high expressing DD genotype however has been associated with a number of adverse outcomes. Marshall et al found that patients on ITU with the DD genotype were significantly more likely to develop ARDS compared to those without DD and this genotype was significantly associated with mortality in the ARDS group (Marshall et al., 2002). The DD genotype has also been associated with a higher Paediatric Risk of mortality (PIM) score, higher Glasgow Meningococcal Septicaemia Prognostic Score (GMSPS) and severity of illness in a paediatric cohort with meningococcal disease (Harding et al., 2002).

Endotoxin

Endotoxin, found in the outer membrane of gram-negative bacteria, is an important trigger of SIRS. With co-factors including CD14 and lipopolysaccharide binding protein (LBP), endotoxin binds to Toll-like receptor 4 (TLR-4), one of a family of transmembrane proteins expressed on key cells including macrophages, endothelial cells and neutrophils. TLR-4 recognises 'pathogen-associated molecular patterns' a key event in the innate immune response (Stephens & Mythen 2000). TLR-4 stimulation initiates a complex series of intra-cellular signalling events (involving MyD88, IRAK, TRAF6, and NF-κB) resulting in the production of pro-inflammatory mediators including cytokines and adhesion molecules. Experimentally endotoxin can initiate a systemic inflammatory response and is found in large quantities in the colonized human gut (Suffredini et al., 1999). Critically ill patients, as well as those undergoing surgery, may be exposed to endotoxin from leakage into the systemic circulation via an impaired gastrointestinal barrier, gram-negative infection or as a result of bowel manipulation during surgery.

All adult humans have antibodies directed against the core of endotoxin (EndoCAb) although observed levels vary within populations by more than eighty-fold (Stephens &

Mythen 2000). IgG EndoCAb is present at birth, and is probably maternal in origin (trans-placentally acquired). IgM EndoCAb is almost absent in the first month but increases to approximately adult levels by a year (Oppenheim et al., 1994). In adults, higher pre-operative levels of IgM EndoCAb are associated with a good outcome following surgery, whilst higher IgG EndoCAb levels have been linked to survival in sepsis (Bennett-Guerrero et al., 2001b; Bennett-Guerrero et al., 2001a; Goldie et al., 1995; Strutz et al., 1999). It is not known if this is a causal association with EndoCAb acting to modulate systemic inflammation, or if high EndoCAb titres are simply a consequence of a favourable immune state in patients at risk of systemic inflammation.

The aims of this part of my work were:

1) to investigate whether children who develop SIRS or severe sepsis in their initial period on ICU have lower levels of antibodies to endotoxin core,

2) to determine whether polymorphisms in one of the genes for TNF-α, IL-6, IL-10, angiotensin converting enzyme (ACE) and plasma activator inhibitor 1 (PAI-1) may also play a role in the development of SIRS and

3) to determine if the association observed in chapter 6 that *MBL-2* polymorphisms are associated with the development of SIRS and/or sepsis is confounded or influenced by these other key polymorphisms and/or antibodies to endotoxin core?

7.2 Methods

7.2.1 Patient Selection

These patients are the same as those in the previous chapter- see section 6.2.1

7.2.2 Consent & recruitment, ethical approval, blood sample processing

These patients are the same as those in the previous chapter- see section 6.2.2-6.2.5.

Blood serum samples were initially divided thus allowing a second sample for EndoCab analysis which had had remained frozen or had at most one thaw/refreeze.

7.2.3 EndoCAb ELISA

Polystyrene microplates (pre-coated with an equimolar mixture of incomplete core, rough, mutant endotoxins from each of four species of gram-negative bacteria complexed with polymyxin B were used to measure IgM and IgG EndoCAb concentrations using an enzyme-linked immunosorbent assay described previously (Barclay & Scott 1987). An eight-point standard curve was constructed using doubling dilutions of a pooled-serum calibrated in EndoCAb median units, where 100 is the median value for 1,000 healthy adults' immunoglobulin G or immunoglobulin M, respectively. Test and control samples were diluted 1:200 with dilution buffer and 100 μL of each sample to be assayed added in duplicate to the pre-coated plate and incubated for 1 h at 37°C. After washing 3 times with wash buffer (sodium chloride, 0.138 M; phosphate, 0.01 M; pH 7.4 containing 0.10% [v/v] polyoxyethylene sorbitan monolaurate), 100 μL of a diluted alkaline phosphatase conjugated goat antihuman IgG or IgM antibody (Sigma-Aldrich Chemical Co; Poole, UK) was added to each well. After incubation for 1 h at 37°C the plates were washed three times with wash buffer then once with distilled water, and blotted dry. Substrate (180 μL per well), comprising

1 mg/mL disodium p-nitrophenyl phosphate dissolved in 1 M Diethanolamine buffer with 0.5mM magnesium chloride, was added and the plate incubated at room temperature in the dark for 20-30 minutes. The reaction was stopped with 50μL per well of 2M Sodium hydroxide and read at 405-nm wavelength with an automated plate reader (Dynatech MRX, Virginia, USA). Results from the whole plate were rejected and repeated if predetermined characteristics were not met, specifically if the optical density reading was not on a specific part of the standard curve or if the coefficient of variation between readings was greater than 10%.

7.2.4 Polymerase chain reaction (PCR) of e genes for TNF-α, IL-6, IL-10, ACE and PAI-1

7.2.4.1 Preparation of DNA

DNA was extracted previously as part of the MBL study (see chapter 2.3.3.1). To minimize the risk of contamination of stock DNA and facilitate a high throughput, arrays were created of two 96 well plates, each well containing 25μl/well. Each array was clearly labelled and had a corresponding grid sheet listing samples by study number within an 8x12 grid (Fig 7.1).

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	9	17	25	33	41	49	57	65	73	81	89
B	2	10	18	26	34	42	50	58	66	74	82	90
C	3	11	19	27	35	43	51	59	67	75	83	91
D	4	12	20	28	36	44	52	60	68	76	84	92
E	5	13	21	29	37	45	53	61	69	77	85	PC
F	6	14	22	30	38	46	54	62	70	78	86	Blank
G	7	15	23	31	39	47	55	63	71	79	87	Blank
H	8	16	24	32	40	48	56	64	72	80	88	Blank

Fig 7.1 An example of a standard array grid sheet. Each plate contained at least 3 blanks (negative controls) and space for a positive control (PC). 2 array plates were made for the 142 patients. Each number corresponded to an individual patient.

7.2.4.2 Preparation of samples for PCR

Working arrays were defrosted and centrifuged at 1450g for 1 minute. This minimised DNA loss or cross-well contamination on removal of the array lid. Two microlitres of each DNA sample was removed and transferred into a 96-well polycarbonated PCR plate using a multichannel Finnipipette. Positive and negative controls were used in each array to ensure accuracy. Extreme care was taken to ensure that samples were placed in the identical orientation as in the original array. Loaded PCR plates were then centrifuged at 160g for 1 minute to ensure the DNA was at the bottom of each well, before drying on a Thermal Cycler block at 80°C for 10 minutes.

Polymerase chain reactions were performed in a total volume of 20µl. Each reaction was prepared on ice and contained 1x concentration of polmix (50mM KCl, 10mM Tris-HCl (pH 8.3), 0.2mM dATP, dGTP, dTTP and dCTP), 1.5-2.5mM $MgCl_2$, 600-1000pmol of forward and reverse primers, and 25-30 units of *Taq* polymerase. The concentration of each had been optimised prior to the initiation of this work.

The PCR mix was added to each well of the PCR plate using an automatic repeating dispenser. Each sample was overlaid with 20µl of paraffin oil to prevent evaporation. PCR amplification was performed on an MJ Tetrad DNA engine Thermocycler, using cycle conditions specific to each PCR. Presence of product was confirmed on a 2% agarose gel stained with ethidium bromide. PCR cycle conditions for each polymorphism are shown in Table 7.1. Primer pairs to amplify the appropriate region of interest were obtained from the literature and were kindly provided by Dr Paul Kotwinski (shown in Table 7.2).

Polymorphism	Step 1 denature	Step 2	Step 3	Step 4	No. of cycles (steps 2-4)	Termination step
IL-6 -174 G/C	95°C 4 min	95°C 40 sec	50°C 30 sec	72°C 1.5 min	35 cycles	72°C 5 min
IL-10 -1082 G/A	95°C 5 min	95°C 45 sec	59.8°C 45 sec	72°C 45 sec	30 cycles	72°C 10 min
TNF-α -308 G/A	95°C 5 min	95°C 45 sec	57°C 45 sec	72°C 45 sec	30 cycles	72°C 10 min
ACE D/I	95°C 5 min	95°C 45 sec	54 °C 45 sec	72°C 30 sec	35 cycles	72°C 5 min
PAI 1 -675 4G/5G	95°C 5 min	95°C 30 sec	60 °C 30 sec	72°C 30 sec	30 cycles	72°C 2 min

Table 7.1 PCR cycle conditions for each polymorphism

PCR's for each of the 5 polymorphisms were carried out separately.

Polymorphism	Oligonucleotide Sequence 5'-3' (Forward / Reverse)
IL-6 -174	5'-TGA CTT CAG CTT TAC TCT TGT-3'
G/C	5'-CTG ATT GGA AAC CTT ATT AAG-3'
IL-10 -1082	5'-AAT CCA AGA CAA CAC TAC TAA GGC-3'
G/A	5'-CTG GAT AGG AGG TCC CTT AC-3'
TNF-α -308	5'-GGC CAC TGA CTG ATT TGT GTG T-3'
G/A	5'-CAA AAG AAA TGG AGG CAA TAG GTT-3'
ACE	5'- CAT CCT TTC TCC CAT TTC TC-3
D/I	5'- ATT TCA GAG CTG GAA TAA AAT T-3'
PAI 1 -675	5'CACAGAGAGAGTCTGGCCACGT
4G/5G	5'CCAACAGAGGACTCTTGGTCT

Table 7.2 Primer pairs for amplification of DNA

Forward and reverse primers are detailed for each of the polymorphisms detected.

7.2.4.3 Restriction Enzyme Digest

Restriction enzymes are enzymes derived from bacteria that will cleave double stranded DNA at a particular sequence. The restriction enzyme is sensitive even to a single base change in the recognition sequence, and thus can be used to detect point mutations and single base polymorphisms. A single base change, such as those seen in the promoter polymorphisms studied, eliminate or create a cutting site for the restriction enzyme.

Following generation of the PCR product, a restriction enzyme digest mix was made containing sufficient enzyme to digest the PCR product. 8-10μl of PCR product was added to 5μl of digest mix in a 96-well microtiter plate, and the plate centrifuged at 210g for 1 minute. The PCR/digest mix was incubated for a minimum of 4 hours at a temperature appropriate for the specific enzyme used (Table 7.3).

Polymorphism	Restriction Enzyme	Manufacturer	Temperature of digest	Fragments generated (base pairs =bp)
IL-6 -174 G/C	Nla III	New England Biolabs	37°C	G – 190 bp C – 143 / 47 bp
IL-10 -1082 G/A	EcoN1	New England Biolabs	37°C	G – 253 / 97 / 27 bp A – 280 / 97 bp
TNF-α -308 G/A	Ncol 1	New England Biolabs	37°C	G – 210 / 23 / 20 bp A – 233 / 20 bp
ACE D/I	Bsl 1	New England Biolabs	37°C	I 65bp D 85bp
PAI 1 -675 4G/5G	Bsl 1	New England Biolabs	55 °C	-675 4G uncut 98bp -675 5G cuts to 77+22bp

Table 7.3 Details of Restriction Digests

The details of the specific restriction enzyme and optimal temperature for digest are outlined for each cytokine polymorphism. The size of fragments generated are tabulated in the last column.

7.2.5 Microtitre Array Diagonal Gel Electrophoresis (MADGE)

Following PCR and restriction digest, identification of various polymorphisms was conducted. Detection of the promoter polymorphism for IL-6 -174 (G/C) had been previously established using Microtitre Array Diagonal Gel Electrophoresis (MADGE) by the Cardiovascular Genetic laboratory at The Rayne Institute, University College London (Brull et al. 2001;Fishman et al. 1998). This technique had then been optimised and extended for the detection of the IL-10 -1082 (G/A), TNF-α -308 (G/A), ACE and PAI 1 promoter polymorphisms prior to the onset of this study (see acknowledgements section).

Using a 96 diagonal well polyacrylamide gel, with a horizontal current allowed 96 sample to be run on the same gel. For example for IL-10 -1082 the following procedure was performed (as shown in Fig 7.2 & 7.3). Following digestion with EcoN1 overnight at 37°C, 5μl of the PCR product was added to 2μl of formamide dye and loaded onto a 7.5% polyacrylamide gel. The gel was electrophoresed at 100V to separate digest products and visualised using UVP Gel Documentation System. The digestion enzyme EcoN1 cuts the PCR product 3 times if guanine is present at the -308 position, but only twice if there has been a base change to adenine. Other polymorphisms were run in a similar manner.

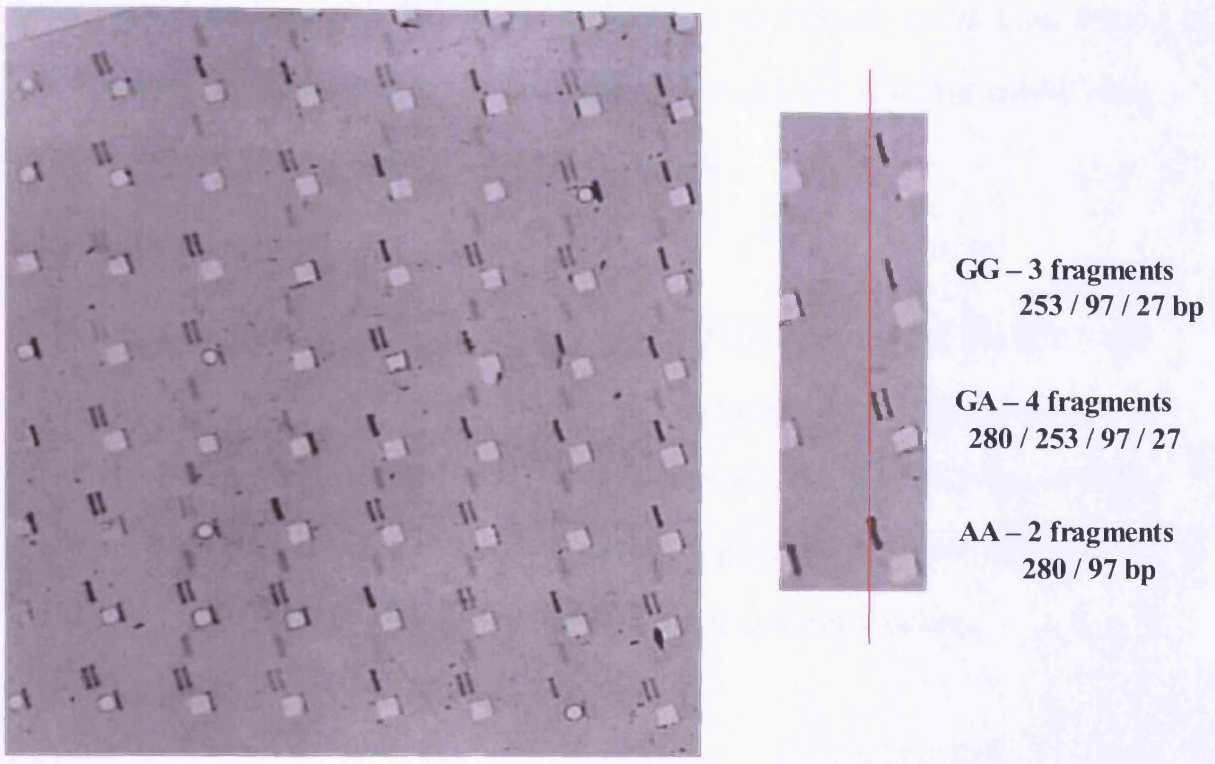


Fig. 7.2 Microtitre Array Diagonal Gel Electrophoresis (MADGE) for IL-10 (-1082 G/A) promoter polymorphism. Following digestion with EcoN1 overnight at 37°C, 5µl of the PCR product was added to 2µl of formamide dye and loaded onto a 7.5% polyacrylamide gel. The gel was electrophoresed at 100V to separate digest products and visualised using UVP Gel Documentation System. The digestion enzyme EcoN1 cuts the PCR product 3 times if guanine is present at the -308 position, but only twice if there has been a base change to adenine. (Gel reproduced with permission from Dr Meredith Allen).

The investigators performing the MBL genotype and serum levels, the cytokine genotypes and the EndoCAb ELISAs were blinded to the diagnosis of SIRS/non SIRS. Likewise the clinician who acquired the clinical data did so before the results were analysed and was therefore also blinded to the outcome.

7.2.6 Statistical analysis

Comparisons of the proportions of different MBL, TNF-α, IL-6 and IL-10, PAI 1 and ACE genotypes between clinical groups were performed using Chi square tests. Multiple and binary logistic regression were used to investigate the association of SIRS and sepsis with MBL serum level & MBL genotype after adjustment for TNF-α, IL-6, IL-10, PAI 1 and ACE genotypes and differences in age, sex and ethnicity.

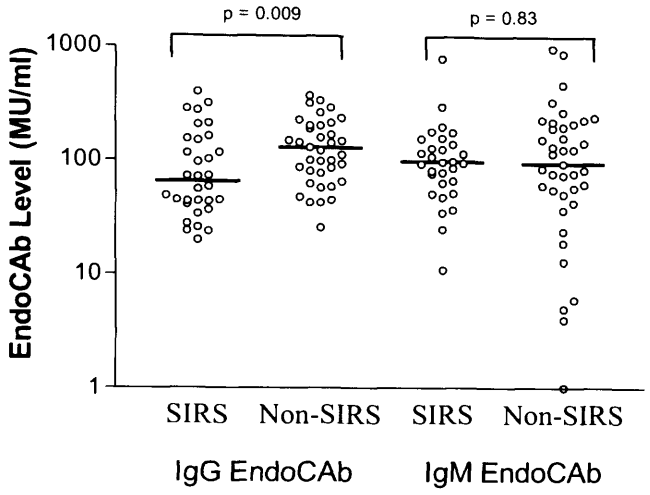
7.3 Results

7.3.1 The effect of MBL on the development of SIRS is not affected by endotoxin immunity

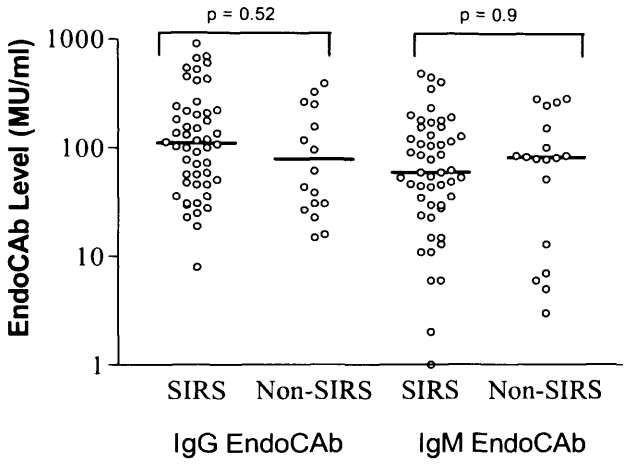
ELISA's for IgM and IgG endotoxin core antibodies (EndoCab) were performed on all 139 serum samples. 71/139 samples were from children admitted without infection and 68/139 from children with infection. Patient demographics were the same as shown for MBL (section 6.3.1). In the unselected cases (n=139), there was a non-significant trend towards lower IgG EndoCAb levels in those patients who had early SIRS, but this did not reach significance; (SIRS median: 99 MU/ml, IQR 44-198 vs Non-SIRS 119 MU/ml, 59-229; p=0.215). There was no difference in the IgM levels between the two groups (87 MU/ml, 44-152 vs 86 MU/ml, 45-189 p=0.588).

In those children without infection, IgG EndoCAb levels were significantly lower in those children developing SIRS (p=0.009 Mann-Whitney U) but there was no difference in IgM EndoCAb levels between the two groups (p=0.829 Mann Whitney U) (Fig 7.3 A) The likelihood ratio for development of SIRS if the IgG EndoCAb was below 57 MU/ml is 3.65 (95% confidence interval 1.48-8.9) compared to those patients with an IgG EndoCAb above 57 MU/ml. Of the potential confounding variables considered (age, sex, ethnicity, CRP & MBL) only MBL level was significantly associated with the development of SIRS on univariate analysis. After binary logistic regression analysis with correction for the effects of age, sex, CRP & MBL, Log_{10} IgG EndoCAb remained independently associated with the development of SIRS in this population. Likewise, following logistic regression analysis, accounting for the above factors, low producing MBL-2 exon 1 genotypes remained significantly and independently associated with the development of SIRS (p=0.0003).

In those children admitted with infection there were no significant differences in IgG or IgM EndoCAb levels between those children developing SIRS and those not (110 vs 80 MU/ml; p=0.523 and 60 vs 83 p=.906 respectively, Fig 7.3 B). There was also no significant difference in the serum IgM & IgG EndoCAb levels in patients who had increasing severity of infection (localized infection vs sepsis vs septic shock, p=0.4 ANOVA test for both EndoCAb IgG & IgM (data not shown). Of the potential confounding variables (age, sex, ethnicity, CRP & MBL) only low MBL levels were associated with the development of SIRS in this group ($p<0.0001$, Mann Whitney U) as previously reported.



A) Patients without infection



B) Patients with infection

Fig. 7.3 Relationship of IgG and IgM EndoCAb to SIRS.

IgG (left two columns) and IgM (right two columns) EndoCAb serum levels are shown for **A)** 71 non-infected patients and **B)** 68 infected patients according to whether they did or did not develop SIRS in the first 48 hours following admission to PICU. In the non-infected patients **(A)** there was a significant difference in IgG EndoCAb between patients with and without SIRS ($p = 0.009$, Mann-Whitney U test).

7.3.2 The effect of MBL on the development of SIRS is not affected by TNF-α, IL-6, IL-10, PAI 1, or ACE polymorphisms.

140 of the original 142 samples were successfully genotyped for TNF-α, IL-6, IL-10, plasma activator inhibitor 1 (PAI 1) and ACE promoter polymorphisms. The two samples that failed did not amplify by PCR for any of the polymorphisms.

Table 7.4 shows the observed and expected frequencies of all genotypes performed. All were in Hardy-Weinberg equilibrium. Observed genotypes did not differ significantly from those expected, apart from IL-6, where a significantly smaller number of patients than expected possessed the CC genotype and a larger number possessed the GG genotype.

Table 7.5 shows the presence of SIRS in all patients, and in those with a non-infective or infective cause for their admission. From this it can be seen that MBL deficient genotypes remains significantly associated with the development of SIRS in all groups. This relationship remained following logistic regression analysis, accounting for the above 5 polymorphisms, age, sex and race. Interestingly in this paediatric PICU population low IL-10 producers (genotype AA) and high IL-6 producers (genotype GG) were also overrepresented in those patients developing SIRS after a non infectious insult only, ($p=0.046$ and 0.0018 respectively). Following binary regression analyses these relationships were found to be present but independent of the MBL effect.

Genotype	Predicted frequency (%)	Observed frequency (%) in PICU group (n=140)	Significant or non significant difference in predicted and observed frequencies
MBL [1]			
A/A (high)	59-62.8	64.3	n/s
A/O (intermediate)	34.4-35.8	34.3	n/s
O/O (low)	2.8-4.6	1.43	n/s
A/O & OO	37.2-40.4	35.7	n/s
TNFα -308 [2]			
A/A (high)	1-16	5	n/s
G/A (intermediate)	16-40	26.4	n/s
G/G (low)	57-84	68.6	n/s
IL6 -174[3]			
G/G (high)	31.8 -36	53.6	$p< 0.01$
G/C (intermediate)	45.4-50.8	40.7	
C/C (low)	18.5-31.8	5.7	
IL10 -1082[4]			
G/G (high)	17-21.3	16.4	n/s
G/A (intermediate)	41.2-60	45	n/s
A/A (low)	23-37.5	38.6	n/s
ACE [5]			
D/D (high)	24.1	22.9	n/s
I/D (intermediate)	51.5	47.9	n/s
I/I (low)	24.4	29.3	n/s
PAI 1[6]			
4G/4G (high)	31-41	27.1	n/s
4G/5G (intermediate)	44-47.7	45	n/s
5G/5G (low)	16-21.3	27.9	n/s

Table 7.4 Frequency of expected and observed genotypes for all polymorphisms studied. Apart from IL-6 there was no significant difference between observed genotype frequencies and those expected from published data. Genotype frequencies were taken from a combination of studies as follows. [1] (Mead et al. 1997) (Garred et al. 1999); [2](Reid et al. 2002); [3](Illig et al. 2004;Schluter et al. 2002) [4](Stanilova, Miteva, Karakolev, & Stefanov 2006), [5](Harding, Baines, Brull, Vassiliou, Ellis, Hart, Thomson, Humphries, & Montgomery 2002); [6](Haralambous, Hibberd, Hermans, Ninis, Nadel, & Levin 2003;Menges, Hermans, Little, Langefeld, Boning, Engel, Sluijter, de Groot, & Hempelmann 2001).

Genotype (expected serum phenotype)	All patients				Non-Infection				Infection			
	All No.	*SIRS No.(%)*	*Non-SIR No.(%)S*	*p value* $\chi 2$	*All No.*	*SIRS No.(%)*	*Non-SIRS No.(%)*	*p value* $\chi 2$	*All*	*SIRS*	*Non-SIRS*	*p value* $\chi 2$
	140	*n=81*	*n=59*		*n=73*	*n=33*	*n=40*		*n=67*	*n=48*	*n=19*	
MBL												
A/A (high)	90 (64.3%)	42 (52%)	48 (81%)	0.0003	52	19 (57.6%)	33 (82.5%)	0.019	38	23 (47.9%)	15(78.9%)	0.02
A/O,O/O (intermediate/low)	50 (35.7%)	39 (48%)	11 (19%)		21	14 (42.4%)	7 (17.5%)		29	25 (52.1%)	4 (21.1%)	
TNFα -308												
A/A (high)	7 (5%)	3 (3.7%)	4 (6.8%)	0.57	3	1 (3%)	2 (5%)	0.19	4	2 (4.2%)	2 (10.5%)	0.48
G/A (intermediate)	37 (26.4%)	20 (24.7%)	17 (24.6%)		18	5 (15%)	13 (32.5%)		19	15 (31.2%)	4 (21.5%)	
G/G (low)	96 (68.6%)	58 (71.6%)	38 (64.4%)		52	27 (82%)	25 (62.5%)		44	31 (64.6%)	13 (68%)	
IL6 -174												
G/G (high)	75 (53.6%)	48 (59.3%)	27 (45.8%)	0.22	42	25 (75.8%)	17 (42.5%)	0.0018	33	23 (47.9%)	10 (52.6%)	0.5
G/C (intermediate)	57 (40.7%)	28 (34.6%)	29 (49.1%)		27	5 (15.2%)	22 (55%)		30	23 (47.9%)	7 (36.8%)	
C/C (low)	8 (5.7%)	5 (6.1%)	3 (5.1%)		4	3 (9%)	1 (2.5%)		4	2 (4.2%)	2 (10.5%)	
IL10 -1082												
G/G (high)	23 (16.4%)	11 (13.5%)	12 (20.4%)	0.12	9	2 (6.1%)	7 (17.5%)	0.046	14	9 (18.7%)	5 (26.3%)	0.73
G/A (intermediate)	63 (45%)	33 (40.8%)	30 (50.8%)		35	13 (39.4%)	22 (55%)		28	20 (41.7%)	8 (42.1%)	
A/A (low)	54 (38.6%)	37 (45.7%)	17 (28.8%)		29	18 (54.5%)	11 (27.5%)		25	19 (39.6%)	6 (31.6%)	
ACE												
D/D (high)	32 (22.9%)	20 (24.7%)	12 (20.3%)	0.53	14	7 (21.2%)	7 (17.5%)	0.12	18	13 (27.1%)	5 (26.3%)	0.86
I/D (intermediate)	67 (47.9%)	32 (39.5%)	35 (59.3%)		42	15 (45.4%)	27 (67.5%)		25	17 (35.4%)	8 (42.1%)	
I/I (low)	41 (29.3%)	29 (35.8%)	12 (20.3%)		17	11 (33.3%)	6 (15%)		24	18 (37.5%)	6 (31.6%)	
PAI 1												
4G/4G (high)	38 (27.1%)	20 (24.7%)	18 (30.5%)	0.72	21	6 (18.2%)	15 (37.5%)	0.09	17	14 (29.2%)	3 (15.8%)	0.31
4G/5G (intermediate)	63 (45%)	37 (45.7%)	26 (44.1%)		33	15 (45.4%)	18 (45%)		30	22 (45.8%)	8 (42.1%)	
5G/5G (low)	39 (27.9%)	24 (29.6%)	15 (25.4%)		19	12 (36.4%)	7 (17.5%)		20	12 (25%)	8 (42.1%)	

Table 7.5 Table of relationship of all genotypes studied to the development of early SIRS. All patients with all 6 genotype results are shown n=140 (of possible 142). Patients are then divided into those with an infective or non infective event precipitating admission to PICU. MBL variant alleles remain overrepresented in all groups of patients with SIRS. High IL6 (GG) and low IL10 (AA) producers are significantly overrepresented in those with non infectious SIRS. Multiple logistic regression analysis showed that the effect of MBL is still present even after taking into account age, sex, race and the above 5 additional polymorphisms.

7.4 Discussion

This chapter describes the influence of a range of polymorphisms on the development of SIRS. Logistic regression analysis, accounting for the polymorphisms detailed, and for age, sex and race, demonstrated that low producing *MBL-2 exon 1* genotypes remained the only significant factor for the *overall* development of SIRS (p=0.0003). As expected from the results presented in chapter 6, MBL deficient genotypes remained significantly associated with the development of SIRS in those who were admitted with both infectious (sepsis) and non-infectious aetiologies and with the severity of sepsis.

Interestingly in our paediatric PICU population low IL-10 producers (genotype AA) and high IL-6 producers (genotype GG) were over-represented in those patients developing SIRS, but only after a non-infectious insult, (p=0.046 and 0.0018 respectively). This in part concurs and in part contrasts, with the study by Stanilova which showed an over-representation of the IL-10-1082 A allele in adult ITU patients with sepsis (ie SIRS after an *infectious* insult) compared to controls. In Stanilova's study although the A allele was associated with *susceptibility* to severe sepsis, once sepsis was established then the presence of the G allele was associated with higher IL-10 levels and mortality (Stanilova, et al., 2006). This may reflect differences between children and adults and in particular the reasons for admission to PICU.

Biologically it would seem plausible that low production of the anti-inflammatory IL-10 and over production of the pro-inflammatory IL-6, as detailed in this chapter, should be associated with SIRS, at least in some populations. This is interesting in the light of more recent studies which indicate that excessive anti-inflammation can also be disadvantageous. One such paper looking at children after cardiac bypass surgery demonstrated that pro-inflammatory cytokine production, in ex-vivo LPS stimulated

whole blood, was *lowest* in those with the *highest* post-operative plasma interleukin-10 levels. Patients with high circulating IL-10 had a more complicated post operative course (Allen et al., 2006). The balance between pro and anti- inflammation may be more important than levels per se. For example, children who are high IL-6 producers may be more inclined to develop SIRS which may be detrimental to the patient. However it may be that this potential for high IL6 production keeps the patient alive long enough to actually reach ICU. Future studies will have to address these issues before effective inflammatory modulation is used routinely in patient care.

The data presented here are also the first to investigate the levels of antibodies against endotoxin core in a mixed population of critically ill children. Overall, EndoCab levels were broadly similar levels to those seen in large adult studies (Bennett-Guerrero et al., 2001; Strutz et al., 1999). In addition this work has shown that cases admitted following trauma or surgery (or for other non-infectious reasons), who go on to develop an early systemic inflammatory response, have lower IgG EndoCAb levels than do those who do not develop SIRS. Higher titres of EndoCAb exhibit anti-inflammatory effects *in-vitro,* for example IgG antibodies to endotoxin core increase the uptake of endotoxin by macrophages, opsonize bacteria, attenuate tumour necrosis factor-α production (Burd et al., 1993) and are protective in a lamb *E coli* model of sepsis (Hodgson et al., 1995). Furthermore increasing levels of IgG or IgM EndoCAb are associated with increasing ability to 'neutralise' endotoxin (Bennett-Guerrero et al., 2001). This simple view must be considered with caution because levels of antibodies to endotoxin core fall in the presence of endotoxin (Rothenburger et al., 2001). Therefore low EndoCAb levels may simply reflect recent infection or exposure to endotoxin from other sources such as on-going poor gut perfusion i.e. it may fall further in patients with SIRS. Of course low EndoCAb may be a non-specific marker of illness

rather than high EndoCAb being protective *per se.* While the mechanisms underlying these relationships remain unclear, it is probably appropriate to consider patients with low titres of EndoCAb as having a 'reduced reserve' against further exposure to endotoxin.

The observation from this study, that the risk of SIRS is increased in critically ill children following trauma or surgery with lower EndoCAb levels, provides some support for the idea that high EndoCAb is protective, rather than that low EndoCAb reflects pre-exiting disease, as a high proportion of these patients were previously healthy trauma cases.

The similarity observed in the EndoCAb levels in the infection sub-group, between patients who went on to develop SIRS or not, is also in keeping with studies in adults with sepsis where there is no clear relationship between initial EndoCAb and outcome (Goldie et al., 1995). The initial IgM EndoCAb concentrations in 146 adults with sepsis were higher in survivors in one study although this may have been explained by other factors (Goldie et al., 1995). A subset of patients with very low initial IgG EndoCAb had increased mortality. In 205 ICU adults with sepsis, a clear relationship was seen between low IgM EndoCAb and progression to septic shock, whilst rising IgG EndoCAb values were associated with a positive outcome. A study employing repeated sampling throughout episodes of infection (including mild cases that do not require intensive care) is required to define the relationship between EndoCAb level and progression to SIRS/ severe sepsis in children.

This study has several limitations. Firstly, it was not possible to collect data on the administration of plasma products to these patients. As these contain antibodies in the same range as the donor population this is a potential confounding factor that would tend to reduce the magnitude of any measured differences (Rashid et al., 2004). Plasma

is not administered on our unit other than in the presence of established coagulopathy so this may be a small effect. More difficult is the fact that it was not possible to correct for the volume of resuscitation fluid administered to each individual. High volume administration of colloid or crystalloid is more likely to be required in patients developing SIRS and would be expected to reduce EndoCAb concentration by a simple dilutional effect. However, this is unlikely to be a major factor as on closer inspection of the data in the infected subgroup there was a trend for *higher* EndoCAb concentrations in patients meeting the criteria for SIRS. In addition, EndoCAb levels vary with age in children: IgM and IgG EndoCAb climb from 3 months, reaching adult values by one year. In this study there were no significant differences in age between the group that developed SIRS and those that did not. Furthermore, in the multivariate analysis of the non-infected group, age did not alter the effect of IgG EndoCAb on the development of SIRS. Lastly, numbers were too small to investigate an effect on mortality.

The hypothesis that EndoCAb has a direct protective role by 'mopping-up' endotoxin is appealing but is far from proven. This study opens up the possibility of immunotherapy aimed at reducing systemic inflammation even after the patients have entered the ICU. Many questions remain including defining the factors that determine EndoCAb levels in an individual.

Whatever the mechanisms underlying the complex interplay between all these reported factors in the innate immune response, MBL deficiency remains consistently, and significantly, associated with sepsis and SIRS in many patient populations (Fidler et al. 2004;Garred et al. 2003;Gordon et al. 2006) and in this further extension to a previously reported study remains so after accounting for various cytokine polymorphisms and serum EndoCab levels.

CHAPTER 8

Final Discussion and Future Work

This project was stimulated by the questions; why do individuals differ in their susceptibility to, and severity of, various infectious and non infectious diseases and could the role of inherited factors governing host response to infection and inflammation be important?

Over the last 18 years MBL has been shown to play an important role in innate immunity (Turner, 1996) and deficiency of MBL, due to gene mutations, has been associated with a large number of infectious and autoimmune diseases (Garred et al., 1995;Garred et al., 1997a;Hibberd et al., 1999;Summerfield et al., 1997;Ohlenschlaeger et al., 2004;Garred et al., 1999a). Just prior to the commencement of this work two papers were published which demonstrated that MBL plays a modifier role in adults with cystic fibrosis. In those who were MBL deficient, a faster progression to end stage lung disease (death or transplantation) and a reduction in lung function was seen (Gabolde et al., 1999;Garred et al., 1999b). There were however some discrepancies in whether this effect was seen in both hetero *and* homozygotes for MBL deficiency or just those homozygous/compound heterozygotes for these defects. CF is a disease characterized mainly by lung involvement in which both infection and inflammation play major roles in disease pathophysiology. What was unclear from these two studies was whether MBL played a role by just by influencing the susceptibility to infection or whether it played a more complex role in the host's inflammatory response. In order to look at this in more detail the work described in chapter 3 of this thesis was designed to look at a larger cohort of patients with CF and to include children as well as adults.

This demonstrated, in the largest study to date, that MBL deficiency is associated with worse lung function in adults with 2 structural MBL mutations only. In older children a significantly increased risk of lung transplant or death was seen. However in the overall paediatric population (the youngest patients studied to date) no effect of MBL genotype on lung function was seen, suggesting that the effect of MBL may be masked in childhood whilst lung function is relatively preserved but becomes more apparent in later years with the expected decline in lung function. During the years over which this work was performed a number of other studies have confirmed the importance of MBL as a disease modifier of CF (see table 3.4).

If MBL does play a role in CF and non CF lung disease (see 1.6.1.1) then the most plausible mechanism for this effect would involve the presence of MBL in the airway. MBL had not hitherto been shown to be present in human respiratory secretions. Chapter 4 was therefore designed to answer the question, can MBL reach the airway surface, by looking at the bronchoalveolar lavage fluids of children with acute and chronic lung diseases and in those without lung disease to act as a control group. In this chapter, I have demonstrated that MBL could be detected in the BAL of children with lung disease but none of the controls. In addition, a higher proportion of children with acute lung disease had MBL detected compared to those with chronic disease and MBL levels were highest in those with acute disease.

One of the original hypotheses from Garred's work was that the effect of MBL deficiency on lung disease was due to the role that MBL plays in the susceptibility to infection, specifically *Pseudomonas aeruginosa* and Bcc. In a small number of patients (n=10) with CF and Bcc infection, an overrepresentation of MBL variant alleles was noticed (Garred et al., 1999b). Chapter 5 addressed this in 2 parts. Firstly MBL binding to various organisms from the Bcc complex was determined and it was seen that

although MBL does bind to many Bcc organisms, binding patterns were not genomovar specific. Secondly, MBL genotypes were determined on a large cohort of CF patients infected with Bcc and no overrepresentation of MBL variant alleles were seen. In addition, in both the adult and paediatric CF cohorts no effect of MBL genotype was seen on the rate of, or first age of acquisition of, *Pseudomonas aeruginosa* infection. The implications of these findings are that MBL may not, in this case, be playing a role in governing susceptibility to infection but may be involved in other aspects of disease pathogenesis. In the case of CF, a disease characterized by both infection and inflammation, it seems plausible that MBL may modulate the inflammatory response.

To address this further, in conditions where both infection and inflammation contribute to disease, a cohort of patients who were admitted to PICU with and without infection were followed to see who developed systemic inflammation within 48 hours and this information was correlated with MBL serum levels and gene mutations (Chapter 6). This demonstrated that indeed MBL deficiency, both genotype and phenotype, are significantly associated with the development of systemic inflammation. Understanding the mechanism involved may be explained by work using an *ex-vivo* model, in which it was shown that MBL can modulate production of proinflammatory cytokines from monocytes. Specifically, high levels of MBL (>6000 ng/ml, as demonstrated in some of these PICU pts) were associated with a reduction, and low MBL levels (such as seen in MBL deficiency) with an increase, in these proinflammatory cytokines (Jack et al., 2001b). To look at this in more detail, experiments described in the latter part of chapter 6 were initiated with the aim of looking at the effect of MBL on a wider range of pro *and* anti-inflammatory cytokines, following stimulation of whole blood with a range of organisms seen in sepsis and CF. Unfortunately due to time constraints this piece of work was not completed and will therefore be explored further in future work.

To determine whether the effect of MBL seen on the development of SIRS was solely due to MBL or potentially due to some confounding genes or antibodies known to play a role in the development of inflammation, anti-endotoxin antibodies and a number of gene polymorphisms were investigated (chapter 7). By multiple logistic regression analysis, low producing *MBL-2* exon 1 genotypes remained the only significant factor for the *overall* development of SIRS ($p=0.0003$) after accounting for age, sex, ethnicity, TNF-α, IL-6, IL-10, angiotensin converting enzyme (ACE), plasma activator inhibitor 1 (PAI-1) and antibodies to endotoxin core.

The implications from the work described here, together with that published in the literature suggests that MBL deficiency does in-fact play a role both in the susceptibility to infection and the subsequent inflammatory response.

Mechanisms of MBL's actions

The underlying mechanism by how MBL acts is more fully understood in the pathogenesis of infection than inflammation. Lack of MBL or MBL of lower oligomeric forms in serum, due to genetic mutations, reduces MBL binding to microorganisms thus there is a reduced direct opsonisation. In addition, reduced MBL-MASP interaction and subsequent complement activation will further reduce opsonisation and also terminal complement attack, thus less killing of microorganisms occurs and the risk of certain infectious diseases may increase. MBL deficiency may therefore be an important additional risk factor for infection when there are major predisposing factors (e.g. young age, neutropenia). Intensive care is an environment with many predispositions to infection: breeches in skin and airway, poor nutrition, gut

hypoperfusion and acquired 'immunoparalysis' (Peters et al., 1999). MBL deficiency is likely also to be important in this scenario.

One could postulate that reduced complement activation would reduce inflammatory "byproducts" and thus inflammation should be reduced in patients with MBL deficiency. The study described here with patients in intensive care, however, found the converse and MBL deficiency was associated with an increase in the systemic inflammatory response to both infective (ie sepsis and severe sepsis) and non infective stimuli. This effect of MBL on the development of sepsis has subsequently been confirmed in a number of studies involving adults, and most latterly neonates receiving intensive care (Garred and Madsen, 2004;Garred et al., 2003;Gordon et al., 2006;Eisen et al., 2006;de Beneditti et al., 2007) adding further weight to the suggestion that in some situations MBL also modulates inflammation possibly by modulation of the cytokine response (see full discussion in chapter 6).

Treatment

One patient with CF and end-stage respiratory disease has been treated with intravenous, serum-derived MBL (Garred et al., 2002). A recombinant product designed for systemic use has now passed a phase 1 safety and tolerability trial (Jensenius et al., 2003;Petersen et al., 2006) and trials are planned in the first instance involving neutropenic patients receiving chemotherapy. The data presented in chapter 6, together with the other studies now reported in adult sepsis patients may provide evidence of another group in whom the potential of therapeutic use of MBL is indicated. Additionally the data presented in chapter 4 suggests that MBL can reach the airway and may have a role in host defense directly on the airway surface, which raises the possibility of the topical route as a therapeutic option. Before this can be done, further work needs to be undertaken with *ex-vivo* models to look more closely at the

exact mechanism by which MBL can modulate the inflammatory response. In addition the mechanism and time course of the appearance and breakdown of MBL in the serum and lung, together with how BAL levels may differ over time with specific disease states needs to be explored.

It is hoped that work described here may play a part in the further understanding of the complex nature of the host's inflammatory responses to infectious and non infectious insults. Once understood, further developments in immunomodulatory treatments, that directly benefit patients, may become available.

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